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(54) Title: BACTERIAL PHEROMONES AND USES THEREFOR

(57) Abstract

RP–factors, their cognate receptors, convertases, respective genes and inhibitors or mimetics thereof are described. In particular, antibodies, pharmaceutical compositions and (therapeutic, diagnostic) methods based on the RP–factors and their receptors/convertases are described.

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BACTERIAL PHEROMONES AND USES THEREFOR

Field of the invention

The present invention relates to RP-factors, their cognate receptors, convertases, respective genes and to inhibitors or mimetics thereof. In particular, the invention relates to antibodies, pharmaceutical compositions and (therapeutic, diagnostic) methods based on the RP-factors and their receptors/convertases.

Introduction

Bacterial pheromones

It is known that certain chemicals may mediate intercellular communication in bacterial cultures. Such communication has been shown to be of importance during sporulation, conjugation, changes in virulence and in bioluminescence. It is now clear that a variety of different autocrine and/or paracrine chemical compounds ("pheromones") produced as secondary metabolites are responsible for such social behaviour in prokaryotes (see e.g. Kell et al, 1995, Trends Ecol. Evolution, 10, 126-129).

Pheromones may be distinguished from nutrients inter alia in that: (i) they are produced by the organisms themselves, (ii) they are active at very low concentrations (e.g. at picomolar or nanomolar concentrations), and (iii) with the exception of prohormone processing, their *metabolism* is not necessary for activity (although they may of course ultimately be degraded).

The chemical nature of these pheromonal compounds varies widely: those associated with Gram-negative organisms tend to be of low molecular weight (e.g. N-acyl homoserine lactone derivatives), whilst a number of Gram-positive organisms use proteins and polypeptides (Kell et al,1995, *ibidem*).

Pheromones are also known to play an important role in the development of bacterial cultures. For unstressed (uninjured) bacteria and optimal growth media, the "self-promoting" mode of culture growth is normally masked due to the high rate of production of growth factors and the sensitivity of the cells to these pheromones. Only under unfavourable conditions (for example, poor growth media, small initial inocula and/or starved cells) is this self-promoting behaviour "visible".

For example, a dramatic reduction in the length of the lag phase of cultures of *Nitrosomonas europea* is mediated by N-(3-oxo-hexanoyl) homoserine lactone, and chorionic gonadotropin-like ligand (a 48kD protein) had similar growth-stimulating activity for *Xanthomonas maltophila*. A number of mammalian hormones (including peptide and steroid hormones as well as cytokines) have also been shown to exhibit

potent growth-stimulating activities for both Gram-positive and Gram-negative bacteria.

Latency and resuscitation

The ability of a microbial cell to grow and divide on a nutrient agar plate constitutes the benchmark method for determining the number of living cells in a sample of interest. However, it is also widely recognised that, especially in nature, the distinction between life and non-life is not absolute; many cells may exist in "dormant" or "moribund" forms or states and will not produce colonies on nutrient media (i.e. are "non-culturable"). However, these dormant or latent cells are not dead: they can be returned, by a process known as resuscitation, to a state of viability/ culturability.

For example, it is known that cells of the (high-G+C Gram-positive) bacterium *Micrococcus luteus* can enter a state of true dormancy from which they may be resuscitated by culture supernatants, even in the absence of any 'initially viable' cells.

The latent state has profound medical implications: many pathogenic bacteria (including pathogenic mycobacteria such as *M. tuberculosis*) are known to persist for extended periods in latent states in a host organism. Indeed, tuberculosis is a re-emergent infection of great concern, and it is recognised in particular that the causative organism (*Mycobacterium tuberculosis*) can lie dormant (remain latent) in patients and carriers for periods of years.

The latent state also has important commercial implications, since it complicates many laboratory methods for the detection, cultivation and enumeration of bacteria (for example in the food and healthcare industries).

There is therefore a pressing need to understand the physiological bases of latency and resuscitation.

Summary of the invention

The present invention is based, at least in part, on the discovery of a new class of pheromones which stimulate the resuscitation of bacteria after true dormancy. This "resuscitation factor" (herein embraced by the term "RP-factor") may exhibit activity at picomolar concentrations (implying a non-nutritional role). The elucidation of the structure of the pheromones at the amino acid sequence level has also permitted the present inventors to describe a larger family of proteins, some members of which act more broadly as regulators of cellular growth or replication and not necessarily as resuscitation promoting factors. Further sequence comparisons have also led to the identification of the cognate receptors, at least some of which share certain sequence similarities with their cognate RP-factors.

Thus, in a first aspect of the present invention there is provided an isolated RP-factor.

RP-factors

The term "RP-factor" is used herein to encompass any representative of that family of substances the members of which are capable of resuscitating dormant, moribund or latent cells (e.g. dormant bacterial cells). In addition, the RP-factors of the invention may also exhibit growth-stimulatory activity with respect to growing cells (e.g. growing bacterial cells), and/or may be competent to reduce the lag time of cell (e.g. bacterial cell) cultures. The resuscitation activity (and optionally also the growth-stimulatory activity or lag-time reducing activity) of the RP-factor may be specific for a particular (bacterial) cell (e.g. specific for one or more pathogenic mycobacteria), or may be non-specific. Specificity may be manipulated for example by engineering (e.g. by mutagenesis or chimaerisation, as herein described) of the specificity-determining domain(s) of the RP-factor or by replacement of the signalling domain.

The term "RP-factor" is also used herein in a somewhat broader sense to encompass polypeptides which are expressed by bacteria and which regulate (e.g. promote, trigger, prevent or impair) the growth or multiplication of a cell (the "target cell") by acting as signalling moieties in conjunction with (e.g. by binding to) cognate cellular receptors. Such polypeptides may be referred to herein as bacterial cytokines.

The RP-factors of the invention therefore inlcude bacterial cytokines which may or may not be capable of resuscitating dormant, moribund or latent cells (e.g. dormant bacterial cells) and/or exhibit growth-stimulatory activity with respect to growing cells (e.g. growing bacterial cells). They may or may not also be competent to reduce the lag time associated with the growth of cell (e.g. bacterial cell) cultures. Moreover, some bacterial cytokines which fall within the scope of the term "RP-factor" as defined herein may even prevent or impair the growth of the target cells (particularly where the target cells are eukaryotic (e.g. mammalian) cells).

The RP-factors of the invention may fall into at least two functional classes: autosignalling factors and allosignalling factors. Autosignalling factors act to regulate the growth of the bacterial cell in which they were expressed (i.e. they act as bacterial autocrine factors), while allosignallers act to regulate the growth of other cells (i.e. they act as bacterial paracrine factors). Autosignalling factors therefore act as self-regulators of bacterial cell growth, and may be essential for viability and/or growth. Some RP-factors may function as both auto- and allosignalling cytokines.

Allosignalling factors may exhibit a range of different specificities. Some may act solely on other bacterial cells of the same species as the cell in which they were expressed ("homoactive" factors), while others may act on cells of one or more other species ("heteroactive" factors). Heteroactive factors may exhibit a broad range of specificity: they may act on several different species (for example, in a genus-specific manner), or may be species-specific. Some heteroactive bacterial factors may act on eukaryotic cells, and may be specific for particular cell-types. For example, some heteroactive bacterial cytokines (particularly those produced by certain pathogens) may act on

mammalian cells (e.g. mammalian epithelial, endothelial or immune cells), and may be tissue- or cell-type specific.

Notwithstanding the above explanation, it is postulated that the specificity of at least some RP-factors may be concentration dependent. In these cases, the specificity of any given RP-factor falls within a continuum, so that an autosignalling RP-factor may mediate cross-talk and so exhibit allosignalling activity when present at sufficiently high concentrations. Similarly, allosignalling RP-factors may exhibit homo- or heteroactivity depending on concentration.

The RP-factor may be translocated through the cell membrane, whereafter it may be secreted into the surrounding environment or remain associated with the surface of the cell. Thus, at least two classes of RP-factor may exist: secreted and non-secreted. The secreted RP-factors are characterised by the presence of a secretory signal sequence (the presence of which is readily recognised by those skilled in the art on the basis of the presence of DNA and/or amino acid sequence motifs). The non-secreted RP-factors may be cell-associated or cytosolic factors. Both classes of RP-factor may exist in a single cellular source (e.g. in a single bacterial source). Both classes of RP-factor find application in the invention.

Non-secreted RP-factors may act in at least four different ways: (a) as a membrane-anchored juxtacrine factor mediating a growth regulating signal between two different cells in close physical proximity or contact; and/or (b) as an intercellular signalling moiety upon cleavage by an enzyme (e.g. a convertase, as herein defined) which releases a soluble signalling moiety into the extracellular milieu; and/or (c) as an autocrine factor *via* binding to cognate receptors located on the surface of the cell in which the non-secreted factor is expressed or acting entirely intracellularly; and/or (d) as a cognate receptor for another non-secreted or secreted RP-factor.

Thus, the RP-factors of the invention may include the nine factors identified by the sequences shown in Fig. 1A and the five factors identified by those shown in Fig 1B, together with their species variants, allelic forms, homologues, derivatives, muteins and corresponding secreted/nonsecreted forms (*vide infra*).

Preferably, the RP-factors of the invention are species variants, allelic forms, homologues, derivatives, muteins and corresponding secreted/nonsecreted forms of any one of the nine factors identified by the sequences shown in Fig. 1A and the five factors identified by those shown in Fig. 1B.

The RP-factors may be synthesised in the form of a precursor which is processed to produce a mature form. Such processing may proceed *via* various intermediate (pro-) forms. Such precursors, intermediate forms and mature proteins are all intended to be covered by the term "RP-factor" as used herein, except where indicated otherwise. As used herein, the term "pro-RP-factor" specifically defines any of various precursors (which may or may not be active) of a mature RP-factor.

The processing may comprise proteolytic cleavage and/or secretion. The precursors may be inactive, and become active on processing as a mature form. The precursors may comprise proteins having secretory leader sequences which are removed during secretion (pre- forms). Such forms are herein referred to as "pre-RP-factor or pre-pro-RP-factors". As explained above, such pre- or prepro- forms are also intended to be covered by the term "RP-factor" as used herein, except where indicated otherwise.

Processing may be attendant on the binding of an RP-factor precursor to a cognate receptor. Such receptors may then directly (or indirectly) cleave the precursor to produce a more mature form of the RP-factor. Such processing may occur as a cascade, involving several receptor-processing complexes, and so ultimately result in the production of a mature RP-factor which then acts as a signalling moiety by binding to a terminal (signal transducing) receptor.

In such processing, the proximal (or intermediate) receptors may function as convertases, and the terminal receptor as a signal transducer. However, a receptor may function as both a convertase and a signal transducer. As used herein, the term "convertase" is intended to define a molecule which binds an RP-factor precursor and (directly or indirectly) processes it to produce a more mature form. They may, for example, have protease activity.

The receptors/convertases discussed above may be disposed at the cell surface (e.g. membrane bound), cytosolic or extracellular.

Preferably, the RP-factor is derived from a bacterium (e.g. a pathogenic bacterium). Particularly preferred are RP-factors derived from high G+C Gram-positive bacteria. However, the inventors have also discovered RP-factor family members in representatives of the low G+C Gram-positive organisms, including *Bacillus subtilis* and clostridia. Thus, RP-factors derived from low G+C Gram-positive bacteria (e.g. pathogenic low G+C Gram-positive bacteria) are also preferred according to the invention. Examples of the latter include: *Streptococcus* spp., *Staphylococcus* spp., *Listeria* spp., *Bacillus* spp., *Clostridium* spp. and *Lactobacillus* spp..

The invention also contemplates homologues, allelic forms, species variants, derivatives, muteins or equivalents of the RP-factors and RP-factor receptors/convertases of the invention.

Preferably, the homologues, derivatives, muteins or equivalents of the RP-factor of the invention have at least 20% identity with any one of the particular amino acid sequences shown in Fig. 1A and 1B.

Particularly preferred are homologues, derivatives, muteins or equivalents of the RP-factor of the invention which have at least 30% identity, for example at least 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 98% identity with any one of the particular amino acid sequences shown in Fig. 1A and 1B.

The homologues, derivatives, muteins or equivalents of the RP-factor of the invention may have at least 25% homology with any one of the particular amino acid sequences shown in Fig. 1A and 1B.

Particularly preferred are homologues, derivatives, muteins or equivalents of the RP-factor of the invention which have at least 30% homology, for example at least 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 98% homology with any one of the particular amino acid sequences shown in Fig. 1A and 1B.

The invention also contemplates chimaeric RP-factors. These are factors which comprise one or more heterologous domains. In this context, a heterologous domain is a portion of an RP-factor which is derived from a different RP-factor to that from which the other domain(s) with which it is associated are derived. Such chimaeric RP-factors find particular utility in applications where the specificity and/or activity of the RP-factor is manipulated or altered.

The invention also contemplates all individual functional domains of the RP-factors of the invention as separate and independent entities.

The invention also contemplates recombinant RP-factor. As used herein, the term "recombinant" is intended to define material which has been produced by that body of techniques collectively known as "recombinant DNA technology" (for example, using the nucleic acid, vectors and or host cells described *infra*).

Cognate receptors

In some cases, the cognate cellular receptor is a cell surface receptor: in other cases, it is a cytosolic receptor with which the cytokine interacts after uptake by the target cell. The receptors with which the RP-factors and/or bacterial cytokines of the invention interact may share certain structural motifs with the RP-factors/cytokines themselves. In particular, the receptors may contain a ligand binding domain which is structurally similar to the signalling domain of the cognate RP-factor/cytokine.

The receptors may also comprise a membrane anchor domain and a wall spanning domain.

Preferably, the cognate receptor comprises a receptor domain as hereinbelow defined and/or a wall spanning domain domain as hereinbelow defined and/or a membrane anchor.

Particularly preferred are cognate receptors comprising the amino acid sequence of MtubZ94752 as shown in Fig. 1A or the amino acid sequence of YabE from *B. subtilis* as shown in Fig. 1B.

The cognate receptors may also comprise derivative or equivalent sequences of amino

acid residues which are as defined above but in which amino acids have been added, deleted or substituted (e.g. conservatively substituted), provided that biological activity (e.g. signalling or ligand-binding activity) is substantially retained.

The cognate receptors may also comprise derivative or equivalent sequences of amino acid residues which have at least 20% identity or homology with the amino acid sequence of MtubZ94752 as shown in Fig. 1A or the amino acid sequence of YabE from *B. subtilis* as shown in Fig. 1B, for example at least 30% identity or homology, for example at least 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 98% identity or homology therewith.

RP-factor/cognate receptor domain structure

The RP-factors of the invention (including the bacterial cytokines as also defined herein) and their cognate receptors may comprise a plurality of discrete domains. These domains may be functionally and/or structurally distinct.

The RP-factors of the invention may be characterised by the presence of at least two functional domains: a secretory signal sequence (which may be wholly or partially absent in the active form of the factor) and a signalling domain. The signalling domain may fall into one of at least two distinct classes described in more detail *infra*.

Many RP-factors also comprise a third functional domain which mediates a physical association with the surface of the target cell (hereinafter referred to as the "localizing domain" and described in more detail *infra*).

The RP-factors of the invention may further comprise a specificity-determining domain, which may function in conjunction with the signalling domain.

Non-secreted RP-factors may further comprise a wall-spanning domain (described in more detail *infra*) and/or a membrane anchor.

The gross structure and/or amino acid sequence of the aforementioned domains may vary considerably. In particular, the structure of the surface localizing domain may differ according to the structure of the cell-wall of the target cell. For example, the surface localizing domain may fall into one of at least two distinct classes: class I (which may act on peptidoglycan) and class II (which may act on the outer lipid envelope found in mycobacteria).

The cognate receptors of the invention may be characterised by the presence of at least two functional domains: a receptor domain and a wall spanning domain. They may also comprise a membrane anchor. The receptor domain may be structurally similar to the signalling domain of the cognate RP-factor (as described in more detail *infra*).

Receptor/signalling domain, class I

This domain may be associated with RP-factors from high G+C Gram-positive bacteria (such as mycobacteria and *Micrococcus* spp.) and/or their cognate receptors. When present on RP-factors, the domain may be involved in receptor binding, and may for example bind a structurally similar domain on a cognate receptor. Thus, when present as part of an RP-factor of the invention, the domain is termed the "signalling domain", and when present in the cognate receptor, the domain is termed the "receptor domain".

The domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues indexed by asterisks in any one of the 9 sequences set out in Figure 1A.

In preferred embodiments, the domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues indexed by asterisks and dots in any one of the 9 sequences set out in Figure 1A.

In particularly preferred embodiments, the domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues highlighted in bold upper case type in any one of the 9 sequences set out in Figure 1A.

In more particularly preferred embodiments, the domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues presented in upper case type in any one of the 9 sequences set out in Figure 1A.

The domain may also comprise derivative or equivalent sequences of amino acid residues which are as defined above but in which amino acids have been added, deleted or substituted (e.g. conservatively substituted), provided that biological activity (e.g. signalling or ligand-binding activity) is substantially retained.

The domain may also comprise derivative or equivalent sequences of amino acid residues which have at least 20% identity or homology with any one of the particular amino acid sequences defined above, for example at least 30% identity or homology, for example at least 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 98% identity or homology therewith.

Receptor/signalling domain, class II

This domain may be associated with RP-factors from low G+C Gram-positive bacteria (such as bacilli and clostridia) and/or their cognate receptors. When present on RP-factors, the domain may be involved in receptor binding, and may for example bind a structurally similar domain on a cognate receptor. Thus, when present as part of an RP-factor of the invention, the domain is termed the "signalling domain", and when present in the cognate receptor, the domain is termed the "receptor domain".

The domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues indexed by asterisks in any one of the 5 sequences set out in Figure 1B(B).

In preferred embodiments, the domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues indexed by asterisks and dots in any one of the 5 sequences set out in Figure 1B(B).

In particularly preferred embodiments, the domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues highlighted in bold upper case type in any one of the 5 sequences set out in Figure 1B(B).

In more particularly preferred embodiments, the domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues presented in upper case type in any one of the 5 sequences set out in Figure 1B(B).

The domain may also comprise derivative or equivalent sequences of amino acid residues which are as defined above but in which amino acids have been added, deleted or substituted (e.g. conservatively substituted), provided that biological activity (e.g. signalling or ligand-binding activity) is substantially retained.

The domain may also comprise derivative or equivalent sequences of amino acid residues which have at least 20% identity or homology with any one of the particular amino acid sequences defined above, for example at least 30% identity or homology, for example at least 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 98% identity or homology therewith.

Wall spanning domain

This domain may be associated with non-secreted RP-factors (e.g. cell-associated RP-factors or RP-factors which act as juxtacrine factors) and with the cognate receptors of the RP-factors of the invention. When present, the domain is involved in mediating an interaction with the cell wall such that the RP-factor/receptor as a whole may span it. The wall spanning domain may therefore be bounded by cytosolic and extracellular regions *in vivo*. The domain is often associated with a membrane anchor, the two structural elements acting in concert to maintain the RP-factor/receptor at the cell surface.

The domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues presented in upper case and indexed by hashes (#) in any one of the 5 sequences set out in Figure 1B(A).

In preferred embodiments, the domain may comprise a sequence of amino acid residues,

the identity and relative positions of which correspond to those residues presented in upper case and indexed by hashes and dots in any one of the 5 sequences set out in Figure 1B(A).

In particularly preferred embodiments, the domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues highlighted in bold upper case type in any one of the 5 sequences set out in Figure 1B(A).

In more particularly preferred embodiments, the domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues presented in upper case type in any one of the 5 sequences set out in Figure 1B(A).

The domain may also comprise derivative or equivalent sequences of amino acid residues which are as defined above but in which amino acids have been added, deleted or substituted (e.g. conservatively substituted), provided that biological activity (e.g. signalling or ligand-binding activity) is substantially retained.

The domain may also comprise derivative or equivalent sequences of amino acid residues which have at least 20% identity or homology with any one of the particular amino acid sequences defined above, for example at least 30% identity or homology, for example at least 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 98% identity or homology therewith.

Localizing domain, class I

This domain may be present in secreted RP-factors, and may mediate a physical association with the surface of the target cell by acting to bind peptidoglycan or some other surface component(s). It may therefore act to increase the local concentration of the cytokine at the target cell surface, so promoting activity by increasing the local concentration of RP-factor in the immediate vicinity of the cognate receptor. Localizing domains may therefore be a characteristic feature of allosignalling bacterial cytokines, and may be absent in autosignalling factors or *vice versa*. For example, when present in autosignalling factors, localizing domains may act to retain the factor at or near the cell surface after secretion through the cell membrane.

When present, the localizing domain may confer important binding properties on the RP-factor, whereby binding to cognate receptor is biphasic and characterised by a primary (relatively unspecific) association with the cell surface followed by a secondary (relatively highly specific) association with the cognate receptor.

The domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues indexed by asterisks in any one of the 10 sequences set out in Figure 1C.

In preferred embodiments, the domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues indexed by asterisks and dots in any one of the 10 sequences set out in Figure 1C.

In particularly preferred embodiments, the domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues highlighted in bold upper case type in any one of the 10 sequences set out in Figure 1C.

In more particularly preferred embodiments, the domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues set out in any one of the 10 sequences set out in Figure 1C.

The domain may also comprise derivative or equivalent sequences of amino acid residues which are as defined above but in which amino acids have been added, deleted or substituted (e.g. conservatively substituted), provided that biological activity (e.g. signalling or ligand-binding activity) is substantially retained.

The domain may also comprise derivative or equivalent sequences of amino acid residues which have at least 20% identity or homology with any one of the particular amino acid sequences defined above, for example at least 30% identity or homology, for example at least 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 98% identity or homology therewith.

Localizing domain, class II

This domain may be present in secreted RP-factors, and may mediate a physical association with the surface of the target cell by acting to bind the outer lipid envelope present in mycobacteria. It may therefore act to increase the local concentration of the cytokine at the target cell surface, so promoting activity by increasing the local concentration of RP-factor in the immediate vicinity of the cognate receptor. Localizing domains may therefore be a characteristic feature of allosignalling bacterial cytokines, and may be absent in autosignalling factors.

When present, the localizing domain may confer important binding properties on the RP-factor, whereby binding to cognate receptor is biphasic and characterised by a primary (relatively unspecific) association with the cell surface followed by a secondary (relatively highly specific) association with the cognate receptor.

The domain may comprise an alanine plus proline-rich segment, such as one or more of the amino acid motifs 'A', A, B, B', C, 'C, D, D* and D' (any one of which may be tandemly repeated) as set out in Figure 1D.

In preferred embodiments, the domain may comprise a sequence of amino acid residues corresponding to residues 158-322 of MtubMTV043 as shown in Figure 1D or to that of residues 45-112 of MtubMTV008 as shown in Figure 1A.

The domain may also comprise derivative or equivalent sequences of amino acid residues which are as defined above but in which amino acids have been added, deleted or substituted (e.g. conservatively substituted), provided that biological activity (e.g. signalling or ligand-binding activity) is substantially retained.

The domain may also comprise derivative or equivalent sequences of amino acid residues which have at least 20% identity or homology with any one of the particular amino acid sequences defined above, for example at least 30% identity or homology, for example at least 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 98% identity or homology therewith.

The term "isolated" is used herein to indicate that the factor exists in a physical milieu distinct from that in which it occurs in nature. For example, the isolated factor may be substantially isolated with respect to the complex cellular milieu in which it naturally occurs. The absolute level of purity is not critical, and those skilled in the art can readily determine appropriate levels of purity according to the use to which the factor is to be put.

In many circumstances, the isolated factor will form part of a composition (for example a more or less crude extract containing many other proteins and substances), buffer system or pharmaceutical excipient, which may for example contain other components (including other proteins, such as albumin).

In other circumstances, the isolated protein may be purified to essential homogeneity, for example as determined by PAGE or column chromatography (for example HPLC or mass spectrometry). In preferred embodiments, the isolated RP-factor of the invention is essentially the sole active RP-factor in a given composition. Particularly preferred are compositions in which an RP-factor (or a particular species, homologue, mutein, derivative or equivalent thereof) is present as the sole active ingredient in a pharmaceutical composition.

The RP-factor for use in the invention need not be isolated in the sense defined above, however. For example, more or less crude culture supernatants (e.g. "spent" medium) may contain sufficient concentrations of RP-factor for use in several applications. Preferably, such supernatants are fractionated and/or extracted (see below), but in many circumstances they may be used without pretreatment. They are preferably derived from spent media used to culture RP-factor-producing microorganisms (for example, the bacterial sources described *infra*). The supernatants are preferably sterile. They may be treated in various ways, for example by concentration, filtration, centrifugation, spray drying, dialysis and/or lyophilisation. Conveniently, the culture supernatants are simply centrifuged to remove cells/cell debris and filtered.

Such supernatants find utility in diagnostic kits and methods, for example in the diagnostic kits and methods described *infra*. They also find utility in the recovery from various samples of culturable microorganisms (e.g. from soil, food, marine, freshwater,

or tissue samples) or from samples taken from an organism (e.g. a human or animal).

The culture supernatants may also be used as supplements in various culturing substrates, for example in culture or transport media. The culture medium may take any convenient form, such as for example agar plates, broths, slopes, coated dipsticks, coated probes, membranes, coated or filled wells or films. The medium may be a defined or complex medium, and may contain indicator dyes to facilitate identification of cultured microorganisms. Preferably, the medium is suitable for the culturing or transport of bacteria, for example *Mycobacterium* spp..

The term "isolated" as applied to the other materials of the invention (for example, the genes and other nucleic acids encoding the RP-factor and their cognate receptors/convertases) is to be interpreted mutatis mutandis. Thus, as applied to nucleic acid (e.g. RNA or DNA or (structural) genes), the isolated nucleic acid may be present in any of a wide variety of vectors and in any of a wide variety of host cells (or other milieu, such as buffers, viruses or cellular extracts).

The term "family", as applied to the proteins of the invention, is used herein to indicate a group of proteins which share substantial sequence similarities, either at the level of the primary sequence of the proteins themselves, or at the level of the DNA encoding them. The sequence similarities may extend over the entire protein/gene, or may be limited to particular regions or domains. Similarities may be based on nucleotide/amino acid sequence identity as well as similarity (for example, those skilled in the art recognise certain amino acids as similar, and identify differences based on switches of similar amino acids as conservative changes). Some members of a protein family may be related in the sense that they share a common evolutionary ancestry, and such related proteins may herein be referred to as homologues. The members of a protein family do not necessarily share the same biochemical properties or biological functions, though their similarities are usually reflected in common functional features (such as effector binding sites and substrates).

The criteria by which protein families are recognised are well-known in the art, and include computer analysis of large collections of sequences at the level of DNA and protein as well as biochemical techniques such as hybridisation analysis and enzymatic assays (see for example Pearson and Lipman (1988), PNAS USA, 85: 2444).

Thus, the RP-factors of the invention include the factors shown in Fig. 1A and 1B, together with their species variants, allelic forms, homologues, derivatives, muteins and corresponding secreted/nonsecreted forms (*vide infra*). Preferably, the RP-factors of the invention are species variants, allelic forms, homologues, derivatives, muteins and corresponding secreted/nonsecreted forms of any one of the proteins represented in Fig. 1A and Fig. 1B.

The RP-factors may be synthesised in the form of a precursor which is processed to produce a mature form. Such processing may proceed *via* various intermediate (pro-)

forms. Such precursors, intermediate forms and mature proteins are all intended to be covered by the term "RP-factor" as used herein, except where indicated otherwise. As used herein, the term "pro-RP-factor" specifically defines any of various precursors (which may or may not be active) of a mature RP-factor.

The processing may comprise proteolytic cleavage and/or secretion. The precursors may be inactive, and become active on processing as a mature form. The precursors may comprise proteins having secretory leader sequences which are removed during secretion (pre- forms). Such forms are herein referred to as "pre-RP-factor or pre-pro-RP-factors". As explained above, such pre- or prepro- forms are also intended to be covered by the term "RP-factor" as used herein, except where indicated otherwise.

Processing may be attendant on the binding of an RP-factor precursor to a cognate receptor. Such receptors may then directly (or indirectly) cleave the precursor to produce a more mature form of the RP-factor. Such processing may occur as a cascade, involving several receptor-processing complexes, and so ultimately result in the production of a mature RP-factor which then acts as a signalling moiety by binding to a terminal (signal transducing) receptor.

In such processing, the proximal (or intermediate) receptors may function as convertases, and the terminal receptor as a signal transducer. However, a receptor may function as both a convertase and a signal transducer. As used herein, the term "convertase" is intended to define a molecule which binds an RP-factor precursor and (directly or indirectly) processes it to produce a more mature form. They may, for example, have protease activity.

The receptors/convertases discussed above may be disposed at the cell surface (e.g. membrane bound), cytosolic or extracellular.

Preferably, the RP-factor is derived from a bacterium (e.g. a pathogenic bacterium). Particularly preferred are RP-factors derived from high G+C Gram-positive bacteria.

The term "derived from" as applied to a defined source is intended to define not only a source in the sense of it being the *physical* origin for the material, but also to define material which has structural and/or functional characteristics which correspond to those of material which does originate from the reference source. Thus, a protein "derived from" a given source need not necessarily have been purified from that source.

The term "high G+C Gram-positive bacterium" is a term of art defining a particular class of evolutionarily related bacteria. The class includes *Micrococcus* spp. (e.g. *M. luteus*), *Mycobacterium* spp. (for example a fast- or slow-growing mycobacterium, e.g. *M. tuberculosis*, *M. leprae*, *M. smegmatis* or *M. bovis*), *Streptomyces* spp. (e.g. *S. rimosus* and *S. coelicolor*) and *Corynebacterium* spp. (e.g. *C. glutamicum*). Preferred according to the invention are RP-factors/cognate receptors/convertases derived from mycobacteria ("mycobacterial RP-factors/RP-factor receptors/convertases").

The invention also contemplates homologues, allelic forms, species variants, derivatives, muteins or equivalents of the RP-factors and RP-factor receptors/convertases of the invention.

The term "homologue" is used herein in two distinct senses. It is used *sensu stricto* to define the corresponding protein from a different organism (i.e. a species variant), in which case there is a direct evolutionary relationship between the protein and its homologue. This may be reflected in a structural and functional equivalence, the protein and its homologue performing the same role in each organism.

The term is also used herein *sensu lato* to define a protein which is structurally *similar* (i.e. not necessarily related and/or structurally and functionally equivalent) to a given (reference) RP-factor. In this sense, homology is recognised on the basis of purely structural criteria by the presence of amino acid sequence identities and/or conservative amino acid changes (as set out by Dayhoff *et alia, Atlas of protein structure* vol. 5, National BioMed Fd'n, Washington D.C., 1979).

For the purposes of the invention, homologues may be recognised as those proteins the corresponding DNAs of which are capable of specifically or selectively cross-hybridising, or which can cross-hybridise under selective, appropriate and/or appropriately stringent hybridisation conditions.

The term "selectively or specifically (cross)hybridisable" in this context indicates that the sequences of the corresponding ssDNAs are such that binding to a unique (or small class) of homologous sequences can be obtained under more or less stringent hybridisation conditions. This method of the invention is not dependent on any particular hybridisation conditions, which can readily be determined by the skilled worker (e.g. by routine trial and error or on the basis of thermodynamic considerations).

Preferably, the homologues, derivatives, muteins or equivalents of the RP-factor of the invention have at least 20% identity with any one of the particular amino acid sequences shown in Fig. 1A or Fig. 1B.

Particularly preferred are homologues, derivatives, muteins or equivalents of the RP-factor of the invention which have at least 30% identity, for example at least 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 98% identity with any one of the particular amino acid sequences shown in Fig. 1A or Fig. 1B.

The homologues, derivatives, muteins or equivalents of the RP-factor of the invention may have at least 25% homology with any one of the particular amino acid sequences shown in Fig. 1A or Fig. 1B.

Particularly preferred are homologues, derivatives, muteins or equivalents of the RP-factor of the invention which have at least 30% homology, for example at least 35%,

40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 98% homology with any one of the particular amino acid sequences shown in Fig. 1A or Fig. 1B.

The term "derivative" as applied herein to the proteins (e.g. the RP-factors or RP-factor receptors/convertases) of the invention is used to define proteins which are modified versions of the proteins of the invention. Such derivatives may include fusion proteins, in which the proteins of the invention have been fused to one or more different proteins or peptides (for example an antibody or a protein domain conferring a biochemical activity, to act as a label, or to facilitate purification).

The derivatives may also be products of synthetic processes which use a protein of the invention as a starting material or reactant.

The term "mutein" is used herein to define proteins that are mutant forms of the proteins of the invention, i.e. proteins in which one or more amino acids have been added, deleted or substituted. The muteins of the invention therefore include fragments, truncates and fusion proteins (e.g. comprising fused immunoglobulin, receptor, convertase or enzyme moieties).

The muteins of the invention also include proteins in which mutations have been introduced which effectively promote or impair one or more activities of the protein, for example mutations which promote or impair the function of a receptor, a recognition sequence or an effector binding site.

Muteins may be produced by any convenient method. Conveniently, site-directed mutagenesis with mutagenic oligonucleotides may be employed using a double stranded template (pBluescript KS II construct containing the RP-factor or RP-factor receptor/convertase gene), (e.g. Chameleon™ or QuikChange™ - Stratagene™). After verifying each mutant derivative by sequencing, the mutated gene is excised and inserted into a suitable vector so that the modified protein can be over-expressed and purified.

Preferred mutant forms are truncates consisting (or consisting essentially) of the RP-factor signalling domain or the RP-factor specificity-determining factor, or of the ligand binding domain of the RP-factor receptor, or combinations of two or more of the foregoing.

The invention also contemplates chimaeric RP-factors. These are factors which comprise one or more heterologous domains. In this context, a heterologous domain is a portion of an RP-factor which is derived from a different RP-factor to that from which the other domain(s) with which it is associated are derived. Such chimaeric RP-factors find particular utility in applications where the specificity and/or activity of the RP-factor is manipulated or altered.

Useful in the construction of such chimaeric RP-factors are DNA fragments or cassettes consisting essentially of DNA encoding selected domains (for example, the signalling domain or the specificity-determining domain), the fragment or cassette optionally being bounded by one or more restriction endonuclease cleavage sites or cloning sites. The invention also contemplates concatenated domain cassettes, as well as mutant RP-factor structural genes which have cloning sites (e.g. one or more restriction endonuclease cleavage sites) located in one or more interdomain regions.

The term equivalent as used herein and applied to the materials of the invention defines materials (e.g. proteins, DNA etc.) which exhibit substantially the same functions as those of the materials of the invention while differing in structure (e.g. nucleotide or amino acid sequence). Such equivalents may be generated for example by identifying sequences of functional importance (e.g. by identifying conserved or canonical sequences or by mutagenesis followed by functional assay), selecting an amino acid sequence on that basis and then synthesising a peptide based on the selected amino acid sequence. Such synthesis can be achieved by any of many different methods known in the art, including solid phase peptide synthesis (to generate synthetic peptides) and the assembly (and subsequent cloning) of oligonucleotides.

The homologues, fragments, muteins, equivalents or derivatives of the proteins of the invention may also be defined *inter alia* as those proteins which cross-react with antibodies to the proteins of the invention, and in particular which cross-react with antibodies directed against any of the specific proteins shown Fig. 1A or Fig. 1B.

The invention also contemplates all individual functional domains of the RP-factors of the invention as separate and independent entities.

The invention also contemplates recombinant RP-factor. As used herein, the term "recombinant" is intended to define material which has been produced by that body of techniques collectively known as "recombinant DNA technology" (for example, using the nucleic acid, vectors and or host cells described *infra*).

The invention also contemplates a pharmaceutical composition (e.g. a vaccine) comprising the RP-factor or RP-factor receptor/convertase (or homologue, species variant, allelic form, derivative, mutein or equivalent thereof) of the invention.

A pharmaceutical composition is a solid or liquid composition in a form, concentration and level of purity suitable for administration to a patient (e.g. a human or animal patient) upon which administration it can elicit the desired physiological changes. The vaccines of the invention may include any suitable adjuvant (e.g. Freund's adjuvant, BCG or BCG extracts).

In another aspect, the invention relates to a pharmaceutical composition comprising the material of the invention which is: (a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or (b) in a pharmaceutical excipient, a unit dosage form or in a form

suitable for local or systemic administration.

In another aspect, the invention relates to an antibody (or antibody derivative) specific for the RP-factor (or homologue, derivative, mutein or equivalent thereof) of the invention.

The antibody is preferably in a form suitable for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or formulated in a pharmaceutical excipient, a unit dosage form or in a form suitable for local or systemic administration. The antibody may be labelled and/or immortalised and/or conjugated to another moiety, and such embodiments find particular utility in diagnostic applications.

According to another aspect of the invention there is provided an isolated or recombinant RP-factor receptor.

The receptor/convertase may be derived from any of the sources hereinbefore described, for example from a bacterial source (e.g. a pathogenic bacterial source). Such sources include high G+C Gram-positives, *Micrococcus* spp. (e.g. *M. luteus*); or *Mycobacterium* spp. (for example a fast- or slow-growing mycobacterium, e.g. *M. tuberculosis*, *M. leprae*, *M. smegmatis* or *M. bovis*); or *Streptomyces* spp. (e.g. *S. rimosus* and *S. coelicolor*); or *Corynebacterium* spp. (e.g. *C. glutamicum*).

The invention also contemplates homologues, derivatives, muteins or equivalents of the receptors/convertases of the invention, as well as recombinant RP-factor receptors/convertases (as hereinbefore defined).

The invention also contemplates a pharmaceutical composition (e.g. a vaccine) comprising the receptor/convertase (or homologue, derivative, mutein or equivalent thereof) of the invention.

Preferably, the receptor/convertase (or homologue, derivative, mutein or equivalent thereof) or pharmaceutical composition is: (a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or (b) in a pharmaceutical excipient, a unit dosage form or in a form suitable for local or systemic administration.

Also contemplated is an antibody (or antibody derivative) specific for the receptor/convertase (or homologue, derivative, mutein or equivalent thereof) of the invention. The antibody may be: (a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or (b) in a pharmaceutical excipient, a unit dosage form or in a form suitable for local or systemic administration.

Also contemplated is an RP-factor antagonist or inhibitor.

Preferably, the antagonist or inhibitor comprises: (a) the antibody of the invention; and/or (b) the receptor/convertase of the invention; and/or (c) an RP-factor mutein

comprising an RP-factor specificity-determining domain, which for example lacks a functional signalling domain. The receptor may function as an antagonist or inhibitor if administered in soluble form, where it may act as a sink for soluble RP-factor. Preferably, modified receptors consisting of the receptor domain (and lacking the membrane anchor and wall spanning domain) are used as inhibitors or antagonists. Such derivatives may exhibit higher solubility.

The antagonist or inhibitor of the invention is preferably: (a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or (b) in a pharmaceutical excipient, a unit dosage form or in a form suitable for local or systemic administration.

Also contemplated by the invention is an RP-factor agonist, activator or mimetic. Preferably, the agonist, activator or mimetic comprises: (a) the RP-factor receptor/convertase antibody as herein described; and/or (b) an RP-factor mutein comprising (or consisting of) an RP-factor specificity-determining domain; and/or (c) an RP-factor mutein comprising (or consisting of) an RP-factor signalling domain; and/or (d) operably coupled combinations of any of (a)-(c).

The agonist, activator or mimetic may be: (a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or (b) formulated in a pharmaceutical excipient, a unit dosage form, in a form suitable for local or systemic administration or in admixture with an antibiotic.

Preferably, the agonist, activator or mimetic may be for use in adjunctive therapy (for example formulated or presented in combination with an antimicrobial agent, e.g. an antibiotic).

The invention also contemplates isolated nucleic acid encoding the RP-factor (or homologue, derivative, allelic form, species variant, mutein or equivalent thereof) or RP-factor receptor/convertase (or homologue, derivative, allelic form, species variant, mutein or equivalent thereof) of the invention. The nucleic acids of the invention therefore embrace DNA having any sequence so long as it encodes the proteins of the invention. It will be appreciated by those skilled in the art that as a result of degeneracy in the genetic code, any particular amino acid sequence of the invention may be encoded by many different sequences. Thus, the nucleic acid sequence may be selected or optimised, e.g. with respect to the codon usage in any particular host cell.

The invention also contemplates vectors (e.g. an expression vector) comprising the nucleic acid of the invention. The nature of the vector is not critical to the invention. Any suitable vector may be used, including plasmid, virus, bacteriophage, transposon, minichromosome, liposome or mechanical carrier.

The expression vectors of the invention are DNA constructs suitable for expressing DNA which encodes the desired protein product (e.g. RP-factor or RP-factor receptor) which may include: (a) a regulatory element (e.g. a promoter, operator, activator, repressor

and/or enhancer), (b) a structural or coding sequence which is transcribed into mRNA and (c) appropriate transcription, translation, initiation and termination sequences. They may also contain sequence encoding any of various tags (e.g. to facilitate subsequent purification of the expressed protein, such as affinity (e.g. His) tags).

Particularly preferred are vectors which comprise an expression element or elements operably linked to the DNA of the invention to provide for expression thereof at suitable levels. Any of a wide variety of expression elements may be used, and the expression element or elements may for example be selected from promoters, enhancers, ribosome binding sites, operators and activating sequences. Such expression elements may comprise an enhancer, and for example may be regulatable, for example being inducible (*via* the addition of an inducer).

As used herein, the term "operably linked" refers to a condition in which portions of a linear DNA sequence are capable of influencing the activity of other portions of the same linear DNA sequence. For example, DNA for a signal peptide (secretory leader) is operably linked to DNA for a polypeptide if it is expressed as a precursor which participates in the secretion of the polypeptide; a promoter is operably linked to a coding sequence if it controls the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation.

The vector may further comprise a positive selectable marker and/or a negative selectable marker. The use of a positive selectable marker facilitates the selection and/or identification of cells containing the vector.

Also contemplated by the invention are host cells comprising the vector of the invention. Any suitable host cell may be used, including prokaryotic host cells (such as *Escherichia coli*, *Streptomyces* spp. and *Bacillus subtilis*) and eukaryotic host cells.

In another aspect, the invention provides a culture or transport medium comprising the RP-factor (or homologue, derivative, mutein or equivalent thereof) of the invention. The culture medium may take any convenient form, such as for example agar plates, broths, slopes, coated dipsticks, coated probes, membranes, coated or filled wells or films. The medium may be a defined or complex medium, and may contain indicator dyes to facilitate identification of cultured microorganisms. Preferably, the medium is suitable for the culturing or transport of bacteria, for example *Mycobacterium* spp. *Streptomyces* spp. and *Corynebacterium* spp.

The invention also contemplates a nucleic acid probe comprising nucleic acid complementary to the nucleic acids of the invention. Such probes are preferably selectively hybridisable with nucleic acid encoding the proteins (e.g. the RP-factors of RP-factor receptors/convertases) of the invention. They are conveniently single stranded DNA or RNA probes.

The invention also contemplates a diagnostic kit comprising the factor (or homologue,

derivative, mutein or equivalent thereof), receptor, antibody, probe or culture medium of the invention.

In another aspect, the invention contemplates antisense DNA corresponding to the nucleic acid encoding the RP-factor or RP-factor receptor/convertase of the invention.

The invention also contemplates a process for producing an antimicrobial drug comprising the steps of: (a) providing an RP-factor receptor; (b) providing candidate drugs; (c) screening the candidate drugs by contacting the RP-factor receptor/convertase with one of the candidate drugs and determining the affinity of the candidate drug for the RP-factor receptor, wherein the affinity is an index of antimicrobial activity, and optionally (d) synthesising or purifying a drug having antimicrobial activity on the basis of the identity of the candidate drug screened in step (c).

Preferably, the process for producing an antimicrobial drug comprises the steps of: (a) providing an RP-factor receptor/convertase; (b) providing a candidate drug; (c) providing an RP-factor; (d) screening the candidate drugs by contacting the RP-factor receptor/convertase with one of the candidate drugs in the presence of the RP-factor, and then determining the ability of the candidate drug to compete non-productively with the RP-factor for binding to the RP-factor receptor, wherein the competitive binding ability is an index of antimicrobial activity, and optionally (e) synthesising or purifying a drug having antimicrobial activity on the basis of the identity of the candidate drug screened in step (d).

The invention also covers an antimicrobial drug produced by (or obtainable by) the processes of the invention, and also derivatives thereof.

Also contemplated by the invention is a method for determining the microbiological quality of a product (e.g. a foodstuff, pharmaceutical preparation or medical product) comprising the step of contacting a sample of the product with an RP-factor (for example, an RP-factor as hereinbefore defined). In such methods, the RP-factor preferably forms part of a nutrient composition (e.g. a plate, broth, film or dipstick).

In another aspect, the invention relates to a method of culturing bacterial (e.g. mycobacterial) cells, comprising the step of incubating the cells in a culture medium containing an RP-factor (for example, an RP-factor as hereinbefore defined).

Also contemplated by the invention is an *ex vivo* method of diagnosis, comprising the step of contacting a biological sample with an RP-factor (for example, an RP-factor as hereinbefore defined).

The diagnostic method of the invention preferably includes the step of incubating the culture or transport medium of the invention to permit growth of cells in the biological sample (e.g. bacterial cells).

Also contemplated by the invention is a method of: (a) stimulating the growth of a microorganism; and/or (b) resuscitating a dormant, moribund or latent microorganism; comprising the step of contacting the microorganism with an RP-factor (for example, an RP-factor as hereinbefore defined).

The invention also contemplates a process for producing the recombinant RP-factor or RP-factor receptor/convertase of the invention comprising the steps of: (a) culturing the host cell of the invention, and (b) purifying the factor or receptor/convertase from the cultured host cells (e.g. from a culture supernatant or cell fraction).

Also contemplated by the invention is a process for producing the recombinant RP-factor or receptor/convertase of the invention comprising the steps of: (a) probing a gene library with a nucleic acid probe which is selectively hybridisable with the cognate structural gene to produce a signal which identifies a gene that selectively hybridises to the probe; (b) expressing the gene identified in step (a) (for example by cloning into a host cell, e.g. according to the process as hereinbefore defined) to produce the factor or receptor.

Also covered is a recombinant RP-factor or receptor/convertase obtainable by the above-described process.

Medical applications

The invention permits the isolation, synthesis and rational design of a wide range of novel medicaments and pharmaceuticals for use in therapy, prophylaxis and diagnosis.

The various forms of therapy, prophylaxis and diagnosis in which the materials of the invention find application may involve changing, breaking or perturbing the resuscitation (RP-factor) signal transduction pathway of one or more infecting pathogens.

Thus, the materials of the invention find general application as antimicrobial agents, for example as antibacterial agents. They may therefore be used in the treatment, prophylaxis or diagnosis of microbial (e.g. bacterial) infections, particularly those infections associated with latency (e.g. mycobacterial infections).

Thus, the invention may for example be used to prevent, reduce or interfere with: (a) the resuscitation of a latent (or dormant) pathogen, and/or (b) the growth of a pathogen, and/or (c) the multiplication and spread of a pathogen; and/or (d) the activation of a latent infection (for example a latent bacterial (e.g. mycobacterial) infection).

In general, the materials of the invention may be used to treat conditions in which changing, breaking or perturbing the resuscitation (RP-factor) signal transduction pathway or blockading the RP-factor receptor/convertase associated with an infecting pathogen is indicated.

Particularly useful materials for use in such therapies/prophylactic methods include RP-factor antagonists or inhibitors. Such antagonists or inhibitors may comprise antibodies to the RP-factor or to the RP-factor receptor/convertase as herein defined; the RP-factor receptor/convertase of the invention; an RP-factor mutein, e.g. which comprises an altered RP-factor specificity-determining domain and/or which lacks a functional signalling domain.

RP-factor antibodies act to sequester and ultimately eliminate endogenous RP-factors in a patient bearing a latent microbial infection.

RP-factor receptor antibodies bind non-productively to the receptors associated with the infecting pathogen. Antibodies to the convertase inactivate (e.g. by steric inhibition) the convertase activity and so prevent maturation of the RP-factor. The antibodies may therefore competitively inhibit the binding of endogenous RP-factor to the receptors/convertases associated with the infecting pathogen. Alternatively, they may bind with high affinity (and/or essentially irreversibly) to the RP-factor receptors/convertases and so block RP-factor-ligand binding or RP-factor maturation. A similar activity is displayed by the RP-factor muteins having altered specificity and/or signalling activity.

In either case, the RP-factor-RP-receptor/convertase binding required for resuscitation of latent pathogens, growth of the pathogen and/or progression of the disease state is perturbed, reduced or abolished.

RP-factor receptors for use as therapeutics in such methods are uncoupled from the signal transduction pathway with which they are normally associated. Thus, they are preferably free (i.e. in soluble or dispersible) form and/or not membrane bound. In this way, effective circulating or systemic concentrations of the free RP-factor receptor can be established and maintained in a patient. In this form, the RP-factor receptors act as RP-factor sinks, and titrate out (and preferably ultimately eliminate) endogenous RP-factors in a patient bearing a latent microbial infection. The receptors therefore reduce or prevent activation of the (latent) pathogen and/or stimulation of pathogen growth, so slowing or halting the progression of the infection.

In another aspect, the invention may be used to resuscitate or assist in resuscitating (or activate or assist in activating) a latent (dormant) pathogenic microbe *in vivo* thereby to potentiate adjunctive antimicrobial therapy. The adjunctive antimicrobial therapies for use in such applications are those which depend for full efficacy on a non-latent or active (e.g. growing or replicating) target pathogen population (for example adjunctive therapies based on certain types of antibiotic). Thus, the materials of the invention may act synergistically with various antimicrobial compounds in antimicrobial therapy.

In a preferred embodiment, the invention is used to potentiate the antimicrobial therapy of tuberculosis, for example involving co-administration of one or more of isoniazid, rifampicin, pyrzinamide and/or ethambutol (or streptomycin).

Particularly useful materials for use in such therapies include for example the RP-factors of the invention, RP-factor agonists, activators and mimetics. Such agonists, activators or mimetics may comprise: the RP-factor receptor antibodies as hereinbefore described; the RP-factor convertase as hereinbefore defined; an RP-factor mutein comprising (or consisting of) an RP-factor specificity-determining domain; an RP-factor mutein comprising (or consisting of) an RP-factor signalling domain; and/or operably coupled combinations thereof.

The RP-factor receptor antibodies for use in such methods are those which serve to trigger an efferent signal transduction pathway at the RP-factor receptor. They may therefore act as RP-factor mimetics, breaking latency/dormancy and acting to resuscitate the pathogen.

Particularly useful in such methods are mutant RP-factors having altered specificity (e.g. in which the specificity-determining domain has been mutated or modified). Such mutant RP-factors may be active against a broad range of pathogens (e.g. against substantially all pathogenic or infective mycobacteria) or targeted against specific pathogens (for example, *M. tuberculosis* and *M. leprae*).

The antibodies, RP-factors, receptors and convertases discussed above may be administered directly or *via* a live vaccine vehicle. Such live vaccines vehicles comprise microorganisms which have been genetically engineered to express (and preferably secrete) the therapeutically active antibodies, RP-factors, receptors and convertases of the invention *in vivo*.

The invention therefore finds application in the treatment of a wide variety of microbial infections, and finds particular application in the treatment of latent microbial (e.g. bacterial) infections.

In preferred embodiments, the invention finds application in the treatment of actinomycete or mycobacterial infections, for example those involving *M. tuberculosis, M. leprae, M. bovis, M. kansasii* and *M. avium*.

Other infections which may be treated according to the invention include those involving *Corynebacterium* spp. (including *Corynebacterium diphtheriae*), *Tropheryma whippelii*, *Nocardia* spp. (including *Nocardia asteroides* and *Nocardia brasiliensis*), *Streptomyces* spp. (including *Streptomyces griseus, Streptomyces paraguayensis* and *Streptomyces somaliensis*), *Actinomadura* spp., *Nocardiopsis* spp., *Rhodococcus* spp., *Gordona* spp., *Tsukamurella* spp. and *Oerskovia* spp. as well as other pathogenic organisms from the group referred to as high G+C Gram-positive bacteria. Other infections which may be treated include those involving pathogenic low G+C Gram-positive bacteria (e.g. *Streptococcus* spp., *Staphylococcus* spp., *Listeria* spp., *Bacillus* spp., *Clostridium* spp. and *Lactobacillus* spp.).

The invention may also be embodied in various vaccines or immunotherapeutic agents.

Such vaccines or agents target one or more elements of the RP-factor mediated signal transduction pathway described herein (and in particular, the RP-factor or RP-factor receptors/convertases themselves). Thus, the RP-factors may be administered as part of a vaccine or immunotherapeutic composition to elicit an immune response directed against endogenous RP-factor in the patient, so reducing, preventing activation of the pathogen and so slowing or halting the progression of the infection.

Alternatively (or in addition), the RP-factor receptors/convertases may be administered as part of a vaccine or immunotherapeutic composition to elicit an immune response directed against receptors for pathogen-borne RP-factor in the patient. In this way, cellular and/or humoral immune responses may be stimulated against the pathogen(s) and/or activation of a latent pathogen (or its continued growth or multiplication) *via* the RP-factor signal transduction pathway may be reduced or prevented, so slowing or halting the progression of the infection.

The invention also finds application in the preparation of live vaccines: attenuated microbial strains can be constructed in which the gene(s) encoding (or regulating the expression or activity of) one or more RP-factors are mutated. Such attenuated vaccines may be based on mutant strains of actinomycetes, mycobacteria (for example *M. tuberculosis, M. leprae, M. bovis* (such as *M. bovis* BCG), *M. kanasii* and *M. avium*), *Corynebacterium* spp. (including *Corynebacterium diphtheriae*), *Tropheryma whippelii, Nocardia* spp. (including *Nocardia asteroides* and *Nocardia brasiliensis*), *Streptomyces* spp. (including *Streptomyces griseus, Streptomyces paraguayensis* and *Streptomyces somaliensis*), *Actinomadura* spp., *Nocardiopsis* spp., *Rhodococcus* spp., *Gordona* spp., *Tsukamurella* spp. and *Oerskovia* spp. as well as other pathogenic organisms from the group referred to as high G+C Gram-positive bacteria.

Particularly useful in such attenuated vaccines are strains bearing mutated RP-factor-encoding genes. Such mutations may be frameshift, deletion, insertion and/or substitution mutations. In preferred embodiments the mutations are null mutations (e.g. non-reverting null mutations), and may prevent growth of the microbe (i.e. "attenuate" it). In other embodiments the mutations may result in the expression of mutant RP-factors having altered specificity (e.g. in which the specificity-determining domain has been mutated or modified) and/or which lack a functional signalling domain. Such mutant RP-factors may bind with high affinity (and/or essentially irreversibly) and non-productively to the RP-factor receptors/convertases and so block RP-factor-ligand binding or RP-factor maturation. The attenuated microbial strains of the invention may also bear mutations in other genes (for example, in other genes essential to growth), and may also bear one or more genetic marker elements.

Biotechnological applications

It is widely recognised that the great majority (probably well in excess of 99%) of soil organisms have not yet been cultured. Hitherto uncultured organisms are also expected to exist in other sources. The present invention may be used to permit the recovery of

such organisms by culture from any source. Thus, the invention provides a way of unlocking an immense reservoir of biodiversity that is known to exist, but is presently inaccessible.

Thus, the present invention provides an unprecedented resource from which libraries of potentially useful microorganisms and biomolecules can be generated. Such libraries can then be used in screening methods to search for medically or industrially useful products.

Thus, in another aspect the invention provides a process for producing a library of biomolecules comprising the steps of: (a) providing a sample (e.g. a soil, marine, food, freshwater, tissue or organism-derived); (b) incubating the sample in a culture medium comprising an RP-factor (for example, an RP-factor as defined in the preceding claims or a culture supernatant comprising an RP-factor) to produce a microbial culture; and (c) isolating microorganisms from the culture of step (b).

The process may further comprise the step of screening the isolated microorganisms for those which elaborate one or more biomolecules of interest (for example a metabolite, enzyme, antibiotic (e.g. antiviral, antibacterial or antifungal agent) or toxin).

Also contemplated is a biomolecule produced by (or obtainable by) the above process, or a derivative thereof.

In another aspect, the invention provides a process for producing a library of microorganisms (e.g. bacteria) comprising the steps of: (a) providing a sample (e.g. a soil, marine, food, freshwater, tissue or organism-derived sample); (b) incubating the sample in a culture medium comprising an RP-factor (for example, an RP-factor as defined in the preceding claims or a culture supernatant comprising an RP-factor) to produce a microbial culture; (c) isolating microorganisms from the culture of step (b).

Also contemplated is a microbe produced by (or obtainable by) the above process, or a derivative (e.g. mutant) thereof.

Exemplification

The invention will now be described in more detail with reference to several Examples. These are for exemplary purposes only and are not intended to limit the invention in any way.

Explanation of the Figures

Figure 1: Part A. Multiple sequence alignment of the predicted amino-acid sequences of RP-factor-like gene products from *M. luteus*, *M. tuberculosis*, *M. leprae* and *Streptomyces coelicolor*. Proteins similar to the RP-factor are derived from *M. tuberculosis* (accession nos. U38939,

nt 2406-2765, and Z81368, nt 33932-34396) and *M. leprae* (accession nos. L01095, nt 12292-12759, and L04666, nt 25446-24921). The DNA sequences of interest in accession Z81368 are also encompassed by accession AD000010. N-terminal residues corresponding to predicted Gram-positive signal sequences are underlined. The *M. leprae* L04666 sequence may also contain a short, 32 aa signal peptide.

Part B. Multiple sequence alignment of gene products related to YabE of *Bacillus subtilis*. The alignment is given in two parts (A and B), with aligned residues in upper case. Those residues which are conserved (or conservatively substituted) in two or more sequences are in bold. In Part A, perfectly conserved residues are marked with a hash (#) and conservative substitutions with a dot (.). Cperfring is an incomplete ORF1 from *Clostridium perfringens* (Acc. No. U04966); Caceto506 is an incomplete ORF from contig 506, *Clostridium acetobutylicum* genome sequencing project. YocH from *B. subtilis* and YabE from *B. subtilis* are YocH and YabE predicted gene products from the *B. subtilis* genome sequencing project (Acc. Nos. BG13521 and P37456).

Part C. Alignment of the RP-factor C-terminal domain with known and hypothetical wall-associated proteins from other organisms. Perfectly conserved residues are marked with an asterisk, those conserved in at least 7 sequences are marked with a dot (.).

Part D. Motifs in the C-terminus (residues 158-322) of MtubMTV043.

Part E. Alignment between the predicted amino acid sequence of the *M. luteus* RP-factor and p60 proteins from *Listeria* spp. Many of the residues that are conserved in the alignment between the C-terminal portion of the *M. luteus* RP-factor (residues 125-220) and the *L. monocytogenes* EGD p60 protein (residues 158-245), are also conserved in the p60 protein from six other *Listeria* spp.

Figure 2: Part A. The sequence of the RP-factor-encoding gene and its predicted product. The nucleotide sequence is in lower case with PCR primers in bold. The predicted protein sequence is in upper case bold (single letter code). Protein and peptide microsequence data used for oligonucleotide design are in upper case italics.

Part B. The sequence of a 299 base pair DNA fragment encoding part of an RP-factor from *Streptomyces coelicolor*. The deduced amino acid sequence is given below the DNA sequence using the single letter amino acid code.

Figure 3: The elution profile of the resuscitation activity. Fractions eluted from the DEAE-sepharose column (see Materials and Methods) with 0.25 M KCl were applied to a Mono Q column which was developed with a 20ml linear gradient from 0.08 to 0.28 M KCl in 10 mM Tris-Cl buffer supplemented by 10% glycerol, pH 7.4. 10 ml of a diluted

suspension of starved cells (CFU 3.10^6cells.ml^{-1}, total count $1.2.10^9$cells.ml^{-1}) were added to 200 ml of LMM supplemented with 0.5 % w/v lactate and 0.05% yeast extract containing of 2 µl of each fraction in 5-10 replicates in the Bioscreen instrument. For details see Materials and Methods. **A**: absorbance at 280nm and magnitude of KCl concentration. **B**: resuscitation activity. **C**: SDS-PAGE profile of the fractions following DEAE-cellulose and Mono Q chromatography. Lanes : 1, markers (94,000, 67,000, 43,000, 30,000, 20,100, 14,400); 2, fraction from DEAE-cellulose column; 3, purifed preparation (fraction number 8 from the Mono Q -column). **D**: Reduction of apparent lag phase of viable cells. 10 µl of a diluted suspension of viable, stationary phase cells (viable count 20 cells) was added to 200 µl of LMM supplemented with 0.5 % w/v L-lactate and containing 2 µl of each fraction (from a different experiment to that shown in parts A and B) in 5-10 replicates in the Bioscreen instrument. The apparent lag phase was estimated by extrapolating the exponential growth line to the abscissa.

Figure 4: Effect of purified RP-factor on *M. luteus*.

A. Concentration dependence of RP-factor activity for resuscitation: resuscitation of dormant cells with different concentrations of RP-factor. 10 µl of a diluted suspension of starved cells (CFU 3.10^6 cells.ml^{-1}, total count 5.10^9 cells.ml^{-1}) was added to 200 µl of LMM supplemented with 0.5 % w/v L-lactate, 0.05% yeast extract and RP-factor in concentrations shown in 5-10 replicates in the Bioscreen instrument. For details see Materials and Methods.
B. Growth of washed cells. Stationary phase cells of *M. luteus* grown in LMM were washed five times by suspension and centrifugation in LMM from which lactate had been omitted. Bacteria were finally suspended in the same medium by repeatedly passing them through a syringe, diluted and inoculated into a 20 ml flask containing LMM or LMM plus 31 pM RP-factor. The initial cell density was 250 viable cells per ml and incubation was at 30°C with intensive shaking. Growth was monitored by plating 0.1ml samples on plates containing broth E solidified with agar.

Figure 5: Detection of RP-factor-like genes in *Micrococcus luteus*, *Mycobacterium smegmatis* and *Streptomyces rimosus*.

Part A		Part B	Part C
M. luteus		*M. luteus*	
Lane 1	λ*Bst*EII	λ*Pst*I	λ*Pst*I
Lane 2	*Cla*I	*Xho*I	*S. rimosus Xho*I
Lane 3	*Sal*I	*Stu*I	*S. rimosus Stu*I
Lane 4	*Sac*II	*Sma*I	*S. rimosus Sma*I
Lane 5	*Pst*I	*Pvu*II	*S. rimosus Pvu*II
Lane 6	*Nco*I	*Pst*I	*S. rimosus Pst*I
Lane 7	*Nhe*I	*Kpn*I	*S. rimosus Bam*HI
Lane 8	*Mlu*I	*Bam*HI	*M. smegmatis Xho*I
Lane 9	*Aat*II	λ*Pvu*II	*M. smegmatis Stu*I

Lane 10	*λPst*I	*M. smegmatis Sma*I
Lane 11		*M. smegmatis Pvu*II
Lane 12		*M. smegmatis Pst*I
Lane 13		*M. smegmatis Bam*HI
Lane 14		*λPvu*II

Figure 6 : Effect of *M. luteus* RP-factor on the growth of *Mycobacterium smegmatis* (A) and *Mycobacterium bovis* (B) in batch culture as observed turbidimetrically. *M. smegmatis* was grown in broth E, to which was added RP-factor at 31 pMol/L. Cells were inoculated at a level of *circa* 200 per well, and growth was monitored in the Bioscreen instrument. *M. bovis* was grown in Sauton medium, as described in the Materials and Methods section, to which RP-factor (620 pMol/L) was either added or not. The inoculum was *circa* 1.10^5 cells.ml^{-1}, and the OD shown is the average of 10 separate determinations of 10 separate tubes.

Figure 7: A: Purification of His-tagged RP-factor. RP-factor was expressed in *E. coli* HSM174(DE3) and purified as described *infra*. Shown is the SDS-PAGE profile of fractions following Ni^{2+}-chelation chromatography. The molecular weight (kDal) markers (SIGMA) were bovine serum albumin (67), ovalbumin (43), glyceraldehyde 3-phosphate dehydrogenase (36), carbonic anhydrase (30), soya bean trypsin inhibitor (20.1), and lactalbumin (14.4). Lane: 1, markers; 2, crude extract from *E. coli* containing pET19b vector; 3, crude extract from *E. coli* containing pRPF1; 4, purified recombinant RP-factor.

B: Reduction of the apparent lag phase of viable cells of *M. luteus* by purified recombinant RP-factor. For experimental details see the legend for Figure 3C. A dilution factor of 10^0 corresponds to 33 μg RP-factor/ml.

C: Stimulation of the growth of washed cells of *M. luteus* by purified recombinant RP-factor. Stationary phase cells of *M. luteus* grown in LMM were washed 5 times by suspension and centrifugation in LMM from which lactate had been omitted. Bacteria were finally suspended in the same medium by repeatedly passing them through a syringe, diluted, and inoculated into a 20 ml flask with LMM or LMM in the presence of RP-factor (230 pMol/L). The initial cell density was ca. 10^2 viable cells per ml and incubation was at 30°C with intensive shaking. Growth was monitored by plating 0.1 ml samples on plates containing nutrient broth E solidified with agar.

Figure 8: A: Anti-RP-factor serum inhibits the growth of *Micrococcus luteus*. Bacteria were inoculated at an initial density of $5x10^5$ per ml into lactate minimal medium (LMM) and the OD_{600nm} was monitored at intervals. Growth of the cultures was monitored over 140 hours at intervals. The samples labelled LMM + Ab and LMM + control Ab contain equivalent amounts of immune and pre-immune serum, respectively. Immune serum (Ab) and pre-immune serum (control Ab) were employed at a 1:1000 dilution.

B: RP-factor overcomes the inhibitory effect of anti-RP-factor serum on growth of *Micrococcus luteus*. Bacteria were inoculated at an initial density of 10^7 cells per ml and growth was monitored by measuring the OD_{600nm} at intervals. Immune serum

(Ab) and pre-immune serum (control Ab) were employed at a 1:1000 dilution and RP-factor was added at a final concentration of 50 ng/ml.

Figure 9: Part A. Blocked alignment of nine RP-factors (as explained *infra*, MtubZ94752 may be a cognate receptor). Areas of sequence identity/similarity are indicated by the shaded areas. The *S. coelicolor* gene product shown is a fragment.

Part B. Schematic showing the domain structure of some gene products in the RP-factor family.

Figure 10: Effect of recombinant RP-factor on growth of *M. tuberculosis* in Sauton medium. Sauton medium containing 0.05% Tween-80 and 100μMol/L Na oleate + 10% (v/v) supplement (which contains, per litre, 50g bovine serum albumin, 20g glucose, 8.5g NaCl) was inoculated to an initial cell density of $31x10^3$ cfu/ml (viable count determined by plating on agar-solidified Middlebrook 7H9 medium containing 10% v/v supplement, composition as detailed above) [total count by microscopy = 10^6 cells per ml] with a 2.5 month-old culture of *M. tuberculosis* strain H37Ra grown in the same medium. Growth of tube cultures at 37°C was measured by determining the OD_{600nm} at intervals for 28 days. The undiluted concentrations of the RP-factors, Rpf (*M.luteus*) and Rpf2 (*M. tuberculosis*), employed for these experiments were ca. 10μg/ml.

Examples

Material And Methods

Organisms and media.

Micrococcus luteus NCIMB 13267 (previously described as "Fleming strain 2665") was grown aerobically at 30°C in shake flasks in lactate minimal medium (LMM) containing L-lactate as described previously. When the culture had reached stationary phase agitation was continued at 30°C for up to 2 months. Cultures were then held aerobically at room temperature without agitation for period for up to a further 2-3 months. The apparent initial viability of these cultures at this point (measured by comparing the plate count with the microscopic count) was less than 10^{-3}.

Mycobacterium smegmatis ("fast" strain, All-Russia State Institute for Contol of Veterinary Preparations, Moscow) was grown in either Sauton medium or nutrient broth E (LabM). Overnight pre-cultures were used to inoculate cultures to an initial density of 10^3 cells/ml. *Mycobacterium bovis* (BCG), *Mycobacterium tuberculosis* H37RV and *Mycobacterium avium* were grown in Sauton medium.

M. luteus *Spent medium preparation.*

Supernatant was obtained after the centrifugation of late logarithmic phase *M. luteus* cultures (200-1000 ml) grown in lactate minimal medium or in the same medium in which lactate was replaced by succinate plus 0.01% yeast extract from which macromolecules had been removed by dialysis. The inoculum consisted of 2% of cells grown in rich medium (Broth E, LabM) and then washed in LMM lacking lactate. The supernatants were passed through a 0.22 μm filter (Whatman) before use.

M. luteus *Cell viability by plating.*

Plates consisting of 1.3% Nutrient Broth E (LabM) or lactate minimal medium were used. Cell dilutions were made in quadruplicate with centrifuged and autoclaved spent medium taken from the starved culture. Plates were incubated at 30°C for 3-5 d.

M. luteus *Cell viability by MPN.*

The MPN assay was performed in a Bioscreen C optical growth analyzer (Labsystems, Finland) using lactate minimal medium supplemented by 0.5% lactate and 0.05% of yeast extract as a resuscitation medium. Dilutions of starved cells were made as described. 10 μl of each dilution (5-10 replicates) were added to a well containing 200 μl of either lactate minimal medium supplemented by 0.5% lactate and 0.05% of yeast extract or the same medium with fraction tested (2-20 μl). Growth (optical density) was monitored using a 600 nm filter. Plates were incubated at 30°C with intensive continuous shaking. The overall measurement period was 120h, each well being measured hourly. The fractions obtained after chromatography were dialysed against elution buffer 2 (see below), diluted in resuscitation medium in various proportions (1:10, 1:100, 1:500, 1:1000, 1:5,000, 1:10,000) and filtered through 0.22 μm Gelman filters before testing. The calculation of the MPN was based on published Tables.

Total cell counts

Unstained cells were counted with a phase-contrast microscope and an improved Neubauer counting chamber. In long-term experiments with mycobacteria, organisms were stained with Ziehl-Neelsen reagent before counting.

Chromatography

Pre-wetted DEAE cellulose was added to culture supernatant (1:10 v/v) and incubated at 4°C for 1h with slow stirring. The cellulose was loaded into a column, and washed with 5 volumes of buffer 1 consisting of 10mM Tris-Cl, 1mM EDTA, 1mM DTT, 10% (v/v) glycerol, pH 7.4 with 10mM KCl. The column was eluted stepwise with 2-3 bed volumes of 0.3M KCl in buffer 1. The fraction obtained was slowly diluted with buffer 1 on ice to give a final KCl concentration of 0.08M. Forty column volumes of this fraction was then loaded onto a DEAE-sepharose fast flow column (1 part of sepharose pre-equilibrated

with buffer 1 containing 0.08M KCl). The column was washed with 5 bed volumes buffer 1 containing 0.08M KCl and eluted stepwise with 3 volumes of 0.25M KCl in buffer 1. The fraction obtained was again slowly diluted with buffer 1 on ice to a final KCl concentration of 0.08M, filtered through a 0.22 μm Gelman filter and loaded onto a Mono Q column (model HR5/5, pre-packed, Pharmacia) equilibrated with buffer 2 consisting of 10mM Tris-Cl, 10% glycerol, pH 7.4 containing 0.08M KCl. The Mono Q column was eluted by a linear gradient from 0.08 M to 0.28 M KCl in buffer 2 (the total volume of the elution was 20 ml). The flow rate and fraction size were 1 ml/min and 1ml/tube respectively. All manipulations except the Mono Q chromatography step were performed at 4°C. The fractions obtained were dialysed against 10 mM Tris-Cl containing 10% glycerol (dialysis is important for the retention of activity) and stored at 4°C for up to 5 days without loss of activity. For prolonged storage in a deep freeze, fractions were dialysed in the same way and glycerol added to a final concentration of 20-30% w/v. The protein content in purified preparations was estimated by tryptophan fluorescence using lysozyme as a standard.

Trypsin treatment:

Trypsin was added to the active, dialysed fraction obtained from the mono Q column and diluted by LMM supplemented with 0.5 % w/v lactate and 0.05% yeast extract (1:100) (the final concentration of trypsin was 50 *u*g/ml). The mixture was incubated for 30 min at 37°C. The reaction was stopped by the addition of trypsin inhibitor (100 *u*g/ml). In control experiments trypsin inhibitor was added to the mixture (100 *u*g/ml) prior to incubation.

PAGE electrophoresis.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli. Chromatographic fractions were dialysed against 10mM Tris HCl, pH 7.4 for 4-5 h, dried in a speed-vacuum apparatus (1.5h), dissolved in sample buffer (Sigma, S-3401), loaded onto 15% acrylamide gel and run at a constant voltage of 200V . The gel was stained with colloidal Coomassie G (Sigma).

Chemicals.

Nutrient Broth E, yeast extract and agar were obtained from Lab M, whilst L-lactate (Li salt), succinate, trypsin, soybean trypsin inhibitor and DEAE-Sepharose fast flow were obtained from Sigma. DEAE cellulose DE52 was obtained from Whatman, and Mono S and Mono Q from Pharmacia. Other chemicals were of analytical grade and were obtained from Sigma or BDH.

DNA manipulations.

Protein microsequence data from the N-terminus (**ATVDTWDRLAEexSNGTxD**) and an internal peptide (**VGGEGYPHQASK**) obtained from the purified RP-factor were used to

design two oligonucleotides, denoted A1 [GCSACSGTSGACACSTGGGACCGSCTSGCSGAG] and A2 [GCYTGRTGIGGRTAICCYTCICC], respectively. Taq polymerase was employed under standard conditions to amplify a 147 bp PCR product from *M. luteus* DNA with these primers. The PCR product obtained from *M. luteus* DNA with these two primers was labelled with digoxygenin and used as a probe for Southern hybridisation experiments. *Sma*I-digested genomic DNA was size-fractionated by agarose gel electrophoresis and circa 1.4 kbp fragments were cloned in pMTL20 and established in *E. coli* strain DH5α. Two recombinant plasmids carrying the desired insert were detected by hybridisation, confirmed by PCR using oligonulceotides A1 and A2, and one of them was manually sequenced on both strands using the dideoxy chain termination method.

Standard procedures were employed to isolate DNA from *M. luteus* and *M. smegmatis*. *Streptomyces rimosus* DNA was kindly supplied by Dr. D. Hranueli. Southern hybridisations with *M. smegmatis* and *S. rimosus* DNA were initially carried out under non-stringent conditions (0.5 SSC, 37°C). Stringent conditions (0.1 SSC, 65°C) were subsequently employed for screening an ordered cosmid library of *Streptomyces coelicolor* A3(2) DNA.

Purification of RP-factor

RP-factor purified from culture supernatants of cells grown in lactate minimal medium, according to the protocol described in Materials and Methods, revealed the presence of a significant amount of polymeric material eluted from all types of columns used, which inhibited both the resuscitation of dormant cells and the growth of viable cells of *M. luteus*. Moreover, elevated concentrations of this material could even cause the lysis of cells (not shown). This inhibitory material appears to be a polymer derived from lactate, as lactate-containing LMM stored for 10 hours at room temperature without cells and subjected to the same procedure of purification revealed inhibitory properties similar to those of this spent medium. To avoid this problem we replaced lactate in the growth medium with succinate, although for good growth it proved necessary to add a small amount (0.01 % w/v) of yeast extract dialysed to remove macromolecules.

Using succinate-grown cultures, the active fraction was purified by a combination of anion exchange media (see Material and Methods). The final activity was eluted at around 180 mM KCl from a linear KCl gradient (from 0.08 to 0.28M KCl) on a MonoQ column in 3 adjacent fractions (Fig. 3). It is worth mentioning that it proved important to dialyse the fractions before testing their activity because some fractions were inactive before dialysis. Active fractions did not change their resuscitation activity after dilution up to 400 times (v/v).

Interestingly, those fractions which were active in causing resuscitation could also increase the growth rate of viable cells.

The resuscitation-promoting material from the final purification step was checked by

SDS-PAGE. The final product (Fig. 3C) proved to consist of a single protein with a molecular weight estimated to be ca 16kD. All active fractions consist of single band with maximum content of protein in fraction N9.

Cloning of the RP-factor gene

Two primers were designed from protein microsequence data obtained for the N-terminus of the purified RP-factor and for an internal peptide. They were used to amplify a 147 bp fragment of *M. luteus* DNA, which was cloned and sequenced. The complete gene was then obtained by a combination of inverse PCR using oligonucleotides G1 and G2 and isolation of a 1.4 kbp *Sma*I genomic restriction fragment. Sequencing revealed that the original PCR product was part of a gene capable of encoding a protein having a signal sequence (Fig. 2A). The predicted size of the secreted form of the gene product is 19,148 Dal, and its predicted N-terminal amino acid sequence agrees with the protein microsequence data, including residues that were not used in primer design (Fig. 2A). The fact that the predicted gene product is larger than the RP-factor purified from culture supernatants suggests that it may, for example, be secreted as a precursor which is converted to its biologically active form upon contact with its cognate receptor/convertase.

Identification of RP-factor homologues

A BLAST search was undertaken using the predicted amino acid sequence of the ORF from *M. luteus* as query. Seven genes with substantial similarity have been sequenced previously. Five are found in *M. tuberculosis* and two in *Mycobacterium leprae* (Fig. 1A). One or more gene products in each organism appear to have a secretory signal sequence (underlined in Fig. 1A). The functions of the predicted products of these mycobacterial genes are unknown; they were found by genome sequencing projects. The BLAST search also revealed similarity between residues 126-220 of the RP-factor and a conserved segment of the (major extracellular) p60 proteins that have been implicated in adherence of *Listeria* spp. to 3T6 mouse fibroblasts suggesting, perhaps, a possible role for the RP-factor or a proteolytic product thereof in adhesion in *M. luteus* (Fig. 1E).

In common with *M tuberculosis* and *M. leprae*, *M. luteus* contains a second gene similar to that encoding the RP-factor. Southern hybridisation experiments, using DNA samples cleaved with a range of different restriction enzymes, and the cloned 147 bp fragment as probe (Figs. 5A & B), reveal two hybridising bands. The stronger hybridisation signal arises from the gene encoding the secreted RP-factor. The other gene may correspond to one of the other mycobacterial genes identified above.

Southern hybridisation experiments, using the 147 bp fragment as probe, as well as PCR experiments, using two oligonucleotides based on highly conserved amino acid motifs as primers, indicate that genes encoding proteins similar to the RP-factor are of widespread occurrence, at least throughout Gram-positive bacteria whose DNA has a high G+C content. Similar genes are detectable by either or both of these methods in all six

Streptomyces species we have tested, including *Streptomyces rimosus* (Fig. 5C) as well as in other mycobacteria, including *Mycobacterium smegmatis* (four similar genes - Fig. 5C), *Mycobacterium bovis* (BCG) and *Corynebacterium glutamicum* (2 similar genes).

Domain structure

The sequence information shows that the RP-factor gene and all of its mycobacterial homologues share a secretory signal sequence and a particularly highly conserved, ca. 70-residue segment. One (MTubZ94752) also has a membrane anchoring motif. The conserved 70-residue segment is a candidate for a signalling domain. Most of this segment is weakly hydrophilic (Kyte-Doolittle) and is predicted to form amphipathic α-helical (Garnier-Robson; Chou-Fasman) or β-sheet regions (Eisenberg). Overall, the segment has a low surface probability (Emini). The C-terminal section, by contrast, is much less highly conserved and might be considered a better candidate for determining localization or specificity (i.e. be a cellular compartment-targeting or specificity-determining domain). By analogy with other protein signalling systems (e.g. many pro-hormones in animals, and systemin in plants) it is possible that the proximate signalling molecule is a proteolytically cleaved product.

Two acidic residues, D7 & E13 (numbering according to the *M. luteus* secreted protein), within this segment are absolutely conserved. The KAEQIKRAE segment (residues 51-59) represents an island of particularly high surface probability. These elements may form part of functional domains within the RP-factor protein.

The conserved domain contains four conserved tryptophan residues (one of which is in a region of high surface probability DTWDR - residues 4-8). In the complex between human growth hormone and its first bound receptor, interactions involving two surface-located tryptophan residues in the receptor account for more than 75% of the binding free energy of the complex (Clackson and Wells, Science 267, 383-386, 1995). The two conserved cysteine residues may form a disulphide bridge.

Alignments showing the domain structures of the various proteins are shown in Figs.9A and 9B.

RP-factor activity

As well as resuscitating dormant cells, the purified RP-factor from *M. luteus* has been tested for growth-stimulatory activity against *M. luteus* and several other organisms. It strongly stimulates the growth *of M. luteus* and *M. smegmatis* and it appears to have weaker activity on *M. tuberculosis*, *M. bovis* (BCG) and *M. avium* (see Fig. 6). In all cases, there is a shortening of the apparent lag phase in batch culture (see Figs. 3D, 4B, 6B and Table 1). The factor is active in poor media and in poor media supplemented with yeast extract and it loses activity after boiling or treatment with trypsin.

When ca. 40 pMol/L RP-factor was added to washed cells of *Mycobacterium smegmatis*,

growth occurred after 20-24 hr, whereas the control lacking RP-factor showed no growth after 6 days. Experiments with slowly growing mycobacteria yielded similar results. Growth of *M. bovis* (BCG) was also strongly stimulated by 40 pMol/L RP-factor: growth occurred after 14 days whereas the control lacking RP-factor showed no growth after 90 days. Finally, RP-factor also stimulated the growth of *Mycobacterium tuberculosis*, *Mycobacterium smegmatis*, *Mycobacterium avium* and *Mycobacterium kansasii* (see Table 1).

Table 1. Purified *M. luteus* RP-factor stimulates growth of mycobacteria

Organism	Bacterial growth[$]	
	RP-factor omitted	RP-factor added
Mycobacterium tuberculosis H37Ra	1.3 ± 1.9 (5)	110 ± 32 (5)
Mycobacterium tuberculosis H37Rv	1.5 ± 2 (4)	45 ± 28 (4)
Mycobacterium avium	0 (3)	>300 (3)
M. bovis (BCG)	0 (5)	54 ± 38 (5)
M. smegmatis *	0 (8)	225 ± 44 (8)
Mycobacterium kansasii	2.5 ± 2.5 (3)	90 ± 77 (3)

[$]Growth was estimated microscopically (magnification times 600) after 14 days of incubation; ca. 50 µl of each culture was fixed, stained using Ziehl-Neelsen reagent and counted. Values in the body of the Table are average numbers of cells in a microscope field (10-20 fields counted) ± standard deviation with the number of determinations in parentheses. RP-factor (after elution from the Mono Q column and dialysis) was used at a concentration of circa 40 pMol/L; activity was lost after either trypsin treatment, heating (autoclaving) or filtration through a 12 kDal cutoff membrane.
*Washed cells of *M. smegmatis* were used for this experiment.

Isolation and characterisation of the gene encoding the second homologue from *M. luteus*

A combination of inverse PCR using oligos G1 and G2 (see Fig. 2A) as primers, and cloning of suitably sized genomic restriction fragments, can be employed to isolate the gene encoding the second homologue from *M. luteus*. The sequence of the gene can then be determined, taking care to eliminate any possible PCR errors by analysis of genomic clones and direct sequencing of PCR fragments obtained by combining the products of multiple, independent PCR reactions. Comparative sequence analyses of the proteins from *M. luteus*, *M. leprae* and *M. tuberculosis* can then be used to refine predictions concerning residues, sequence motifs and structural motifs which may be important for biological function.

Over-expression and purification of *M. luteus* and *M. tuberculosis* gene products in *E. coli*

PCR primers can be designed, incorporating suitable restriction sites such that sequences encoding the secreted forms of the *M. luteus* and the *M. tuberculosis* RP-factors can be amplified and inserted, in the correct reading frame, into commercially available plasmids (pET or pCAL vectors). The PCR-amplified fragments can first be cloned in a pBluescript KS II vector (Stratagene) so that their entire sequence can be verified, to eliminate possible PCR errors. (This material can also be employed for site-directed mutagenesis - *vide infra*.) The pET or pCAL constructs can then be employed to obtain controlled expression of large quantities of histidine- or calmodulin binding peptide-tagged proteins that can be purified, essentially to homogeneity, in a single step. Finally, the tags used in protein purification can be removed (using enterokinase or thrombin, as appropriate).

Expression of RP-factor from *Micrococcus luteus* in *E. coli*

Two primers [5'-GTCAGAATTCATATGGCCACCGTGGACACCTGGG-3'] and [5'-TGACGGATCCTATTAGGCCTGCGGCAGGACGAG-3'] were employed to amplify (5 cycles of 30s at 94°C, 30s at 60°C, 30s at 72°C, followed by 15 cycles of 30s at 94°C, 60s at 72°C) the RP-factor coding sequence (i.e. lacking the signal sequence) from the cloned 1.4 kbp *Sma*I fragment of genomic DNA. It was first established in *E. coli* DH5α as a 567 bp *Eco*RI-*Bam*HI fragment in pMTL20 and then excised as a 562 bp *Nde*I - *Bam*HI fragment, inserted into pET19b (Novagen) and re-established in *E. coli* DH5α. The sequence of the PCR product and vector-insert junction in this plasmid, denoted pRPF1, was verified. RP-factor was expressed from RPF1 after transforming it into *E. coli* HSM174(DE3). The protein, containing a His_{10}-tag at the N-terminus, was isolated by sonicating bacteria, previously grown to an $OD_{600nm} = 0.6$ and induced with 0.4 mM IPTG for 4 h, in a modified binding buffer (MBB - 5mM imidazole pH7.9/0.5M NaCl/20mM Tris-HCl/8M urea) containing 5 mM DTT and 2 mM EDTA. After low speed centrifugation, low MW compounds, including EDTA and DTT, were removed by elution through a Sephadex G10 column pre-equilibrated with MBB. A Ni^{2+}-chelation column (Ni^{2+}-coordinated iminodiacetic acid immobilized on Sepharose 6B), was loaded with the G10 eluate, washed with 20 vol MBB and then successively eluted with four 10 vol aliquots of MBB containing 0.01 M, 0.05 M, 0.2 M and 1 M imidazole, respectively. The column was finally eluted with strip buffer (20 mM Tris-HCl, pH 7.9/100 mM EDTA/0.5 M NaCl). Monoclonal anti-(polyHis) antibodies (Sigma, clone His-1) were employed for immunoblot analysis of fractions subjected to SDS PAGE electrophoresis and electroblotted using standard methods. Fractions were dialysed against buffer 2 and assayed for biological activity as indicated above.

Analysis of recombinant RP-factor

The coding sequence corresponding to the secreted form of RP-factor, starting at residue A_{39}, was inserted into pET19b to generate plasmid pRPF1 (*vide infra*). Extracts of IPTG-induced *E. coli* strain HSM174(DE3) containing pRPF1 were challenged with a poly-His

antibody. A strong signal was associated with a protein (apparent size 29 kDal, predicted size 22 kDal) which was eluted from the affinity column by 1M imidazole (Fig. 7A). The His-tagged protein from HSM174(DE3) reduced the apparent lag phase of viable cells of *M. luteus*, whereas the control (material eluted from the same column under the same conditions when an extract from cells containing plasmid vector only was applied) showed no activity (Fig. 7B). The association of biological activity with the recombinant protein, produced in *E. coli* containing pRPF, and the absence of biological activity in the isogenic control containing pET19b, demonstrates unequivocally that the active molecule is indeed a product of the *rpf* gene.

Antibody preparation

A rabbit was immunized three times at one week intervals using recombinant RP-factor (the recombinant protein prepared as described above). The protein was administered at 300 μg of protein per injection in incomplete Freud's adjuvant (0.5 ml protein and 0.5 ml adjuvant) Blood was collected before administration was started and on the 11th day after the last injection. The immunoglobulin fraction was obtained by standard procedures using PEG. Antibodies were additionally purified on a protein G-superose column according to the standard (Pharmacia) protocol. The final protein concentration was adjusted spectrophotometrically to 1 mg/ml.

Alternatively, monoclonal antibodies can be produced using established techniques.

Use of anti-RP-factor antibody to inhibit bacterial growth

Micrococcus luteus was inoculated at an initial density of $5x10^5$ per ml into lactate minimal medium (LMM) and the OD_{600nm} was monitored at intervals. Growth of the cultures was monitored over 140 hours, and the presence of the anti-RP-factor serum (prepared as described above under "Antibody preparation") completely inhibited bacterial growth (see Figure 8).

Expression of a *M. tuberculosis* RP-factor in *E. coli*

Two primers [5'-ATCAGAATTCATATGGACGACATCGATTGGGACGC-3'] and [5'-CGCAGGATCCCCTCAATCGTCCCTGCTCC-3'] were employed to amplify (5 cycles of 30s at 94°C, 30s at 58°C, 30s at 72°C, followed by 25 cycles of 30s at 94°C, 60s at 72°C) the RP-factor coding sequence (i.e. lacking the signal sequence) from *M. tuberculosis* H37Rv genomic DNA. The PCR product was first established in *E. coli* DH5a as a 336 bp *Eco*RI-*Bam*HI fragment in pMTL20 and then excised as a 331 bp *Nde*I - *Bam*HI fragment, inserted into pET19b (Novagen) and re-established in *E. coli* DH5a. The sequence of the PCR product and vector-insert junction in this plasmid, denoted pRPF2, was verified. The *M. tuberculosis* RP-factor was expressed from pRPF2 after transforming it into *E. coli* HSM174(DE3). The protein, containing a His_{10}-tag at the N-terminus, was isolated by sonicating bacteria, previously grown to an OD_{600nm} = 0.9 and induced with 0.4 mM IPTG for 4 h, in binding buffer (BB - 5mM imidazole pH7.9 / 0.5M

NaCl / 20 mM Tris-HCl / 8M urea). After low speed centrifugation, a Ni^{2+}-chelation column (Ni^{2+}-coordinated iminodiacetic acid immobilised on Sepharose 6B), was loaded with the supernatant, washed with 20 vol BB, 20 vol BB containing 100 mM imidazole, and then eluted with 10 vol BB containing 0.5 M imidazole. Additional purification was achieved by MonoQ column chromatography (*vide infra*, save that the salt gradient was from 0.1 M to 1M NaCl). Monoclonal anti-(polyHis) antibodies (Sigma, clone His-1) were employed for immunoblot analysis of fractions subjected to SDS PAGE electrophoresis and electroblotted using standard methods. Fractions were dialysed against buffer 2 and assayed for biological activity as indicated above.

Analysis of a recombinant *M. tuberculosis* RP-factor

The coding sequence corresponding to the secreted form of the *M. tuberculosis* RP-factor (g1655671; acc. no. Z81368), starting at residue D_{50}, was inserted into pET19b to generate plasmid pRPF2 (*vide infra*). Extracts of IPTG-induced *E. coli* strain HSM174(DE3) containing pRPF2 were challenged with a poly-His antibody. A strong signal was associated with a protein which was eluted from the affinity column by 0.5M imidazole. The histidine-tagged protein from HSM174(DE3) caused a slight but significant enhancement of the growth of *M.tuberculosis* H37Rv, as shown in Fig. 10. It also stimulated the growth of *M. luteus* in LMM. The control culture attained a final OD_{600nm} of 1.0, whereas cultures containing the RP-factor (1:100,000 dilution) attained a final OD_{600nm} of between 2.0 and 6.0.

Effect of *M. luteus* RP-factor on growth of *Mycobacterium tuberculosis* cells isolated from macrophages

In three independent experiments, dormant/latent *M. tuberculosis* cells isolated from cultured murine peritonial macrophages were resuscitated by the *M. luteus* RP-factor. The total number of *M. tuberculosis* cells in the heterogeneous suspension obtained from murine macrophages was determined microscopically. The viable cell count was determined by plating on agar-solidified Sauton medium containing 10% (v/v) supplement (which contains, per litre, 50 g bovine serum albumin, 20g glucose, 8.5g NaCl) or by the MPN method, using liquid Sauton medium containing 10% (v/v) supplement (see above).

The viable count (MPN) of these cell suspensions was enhanced between 25 and 2,500 times by the presence of the *M. luteus* RP-factor (added at a final concentration of 10 ng/ml) (see Table 2). All values in the body of the table are numbers of bacteria per ml suspension

Peritoneal macrophages were obtained from white mice (wild type) by a standard protocol. Infection of macrophages by *M. tuberculosis* "Academiya" (laboratory strain) was performed *in vivo* by intraperitonial injection of 10^6 cells (total count) per mouse followed by incubation for 6 days (1st passage). For the second and third passages macrophage cells in monolayers were infected using *M. tuberculosis* cells isolated from

macrophages from the previous passage.

TABLE 2: Effect of *M. luteus* RP-factor on growth of *Mycobacterium tuberculosis* cells isolated from macrophages

Experiment	Total count [x] (determined microscopically)	Viable count (determined by plating)	Viable count (MPN)	MPN in presence of RP-factor
I	$10^6 > x > 10^5$	90	70	4.10^3
II	$10^6 > x > 10^5$	9	40	1.10^3
III	2.10^6	<1	<1	24.10^3

Macrophages were grown as a monolayer on plastic petri dishes (10^6 cells/5 cm^2) in standard RPMI medium containing gentamicin and penicillin (10μg/ml, each) under standard conditions (CO_2/O_2 mixture in a 37°C incubator). *M. tuberculosis* cells were recovered from macrophages by passing them repeatedly through a thin syringe needle. Macrophage cell debris was removed by low speed centifugation and *M. tuberculosis* cells were then collected by centrifugation at higher speed.

Effect of *yabE* and *yocH* knockout mutations on growth of *Bacillus subtilis*

The entire *yabE* coding region together with flanking sequences was amplified from *B. subtilis* genomic DNA using primers D11 [5'-GAAGAGAATTCCTTCCATCACGA-3'] and D12 [5'-CCAAACGAATTCGGTCAATCAC-3'] as a 1803 bp product. A 1186 bp *Hin*dIII-*Bcl*I fragment encompassing the 3' end of the coding sequence was excised from the PCR product, ligated with *Hin*dIII + *Bam*HI-digested pMTL20, and used to transform *E. coli* strain DH5α with selection for ampicillin-resistance. Plasmid pYABE was isolated from one of the transformants. A 763 bp *Hin*dIII-*Bam*HI fragment from entirely within the *yabE* coding sequence was excised from the pYABE, ligated with *Hin*dIII + *Bam*HI-digested pMUTIN4, an integrating plasmid that may be employed for generating knockout mutations in *B. subtilis* (Edwards & Errington, 1997, Molecular Microbiology, 24, 905-915) and used to transform *E. coli* strain XL1-Blue with selection for ampicillin-resistance. Plasmid pYAB2, containing an internal segment of the *yabE* coding sequence, was isolated from one of the transformants. A 1207 bp *Hin*dIII-*Eco*RI fragment encompassing the 3' end of the *yabE* coding sequence was excised from pYABE, ligated with *Hin*dIII + *Eco*RI digested pMUTIN4 and used to transform *E. coli* strain XL1-Blue

with selection for ampicillin-resistance. Plasmid pYAB3, containing the 3' end of the *yabE* coding sequence, was isolated from one of the transformants.

The entire *yocH* coding region together with flanking sequences was amplified from *B. subtilis* genomic DNA using primers D10 [5'-GCAAGGATCCCAGACTAAAAAAACAG-3'] and D9 [5'-ATCAGGATCCATATTATTAGTTTAAGA-3'] as a 1145 bp product. A 358 bp *Hpa*I fragment from entirely within the *yocH* coding sequence was excised from the PCR product, ligated with *Sma*I-digested pMTL20, and used to transform *E. coli* strain XL1-Blue with selection for ampicillin-resistance. Plasmid pYOC2a, containing an internal segment of the *yocH* coding sequence, was isolated from one of the transformants. The insert in this plasmid was then excised from pYOC2a as a 385 bp *Eco*RI-*Hin*dIII fragment and inserted into pMUTIN4, to yield pYOC2. A 307 bp *Hin*dIII-*Bam*HI fragment encompassing the 3' end of the *yocH* coding sequence was excised from the 1145 bp PCR product, ligated with *Hin*dIII + *Bam*HI digested pMUTIN4, and used to transform *E. coli* strain DH5α with selection for ampicillin-resistance. Plasmid pYOC3, containing a DNA segment encompassing the 3' end of the *yocH* coding sequence, was isolated from one of the transformants.

Plasmids pYAB2, pYAB3, pYOC2 and pYOC3 were linearised with *Apa*I, which cleaves once in the pMUTIN4 vector sequences, ligated with T4 DNA ligase and employed to transform *Bacillus subtilis* strain SA253 *nonA nonB leuA8 arg-15* with selection for resistance to erythromycin on a rich nutrient medium (LB + 1 μg Em/ml). Em^R transformants were then picked and verified by Southern hybridization. Using the integrating plasmid as probe, and digesting the chromosomal DNA with *Apa*I, strains harbouring a single copy of the integrated plasmid gave two hybridising bands whereas the wild type (and any spontaneous Em^R mutants that were present) gave a single hybridising band.

Analysis of the products of transformation with each of the four plasmids indicates that *yabE* and *yocH* gene products are required for growth (at least under certain conditions) in *B. subtilis*.

CLAIMS:

1. Isolated RP-factor.

2. The factor of claim 1 which is a secreted RP-factor.

3. The factor of claim 1 which is a non-secreted RP-factor (e.g. a cell-associated or cytosolic factor).

4. The factor of any one of the preceding claims which is derived from a bacterium (e.g. a pathogenic bacterium).

5. The factor of claim 4 which is derived from:

(i) a high G+C Gram-positive bacterium; or

(ii) a low G+C Gram-positive bacterium (for example *Streptococcus* spp., *Staphylococcus* spp., *Listeria* spp., *Bacillus* spp., *Clostridium* spp. and *Lactobacillus* spp.).

6. The factor of claim 5(i) which is derived from:

(a) *Micrococcus* spp. (e.g. *M. luteus*); or

(b) *Mycobacterium* spp. (for example a fast- or slow-growing mycobacterium, e.g. *M. tuberculosis, M. leprae, M. smegmatis* or *M. bovis*); or

(c) *Streptomyces* spp. (e.g. *S. rimosus* and *S. coelicolor*); or

(d) *Corynebacterium* spp. (e.g. *C. glutamicum*).

7. A homologue, derivative, allelic form, species variant, mutein or equivalent of the factor of any one of the preceding claims.

8. A factor of any one of the preceding claims which comprises (or consists of) the RP-factor signalling domain.

9. A factor of any one of the preceding claims which comprises (or consists of) the RP-factor specificity-determining domain.

10. Recombinant RP-factor, wherein the RP-factor is for example as defined in any one of the preceding claims.

11. A pharmaceutical composition (e.g. a vaccine) comprising an RP-factor as an active ingredient (for example the factor (or homologue, derivative, allelic form, species variant, mutein or equivalent) as defined in any one of the preceding claims), the RP-factor for example being present at a concentration sufficient to confer biological activity on the pharmaceutical composition.

12. An RP-factor (for example the factor (or homologue, derivative, allelic form, species variant, mutein or equivalent) or pharmaceutical composition as defined in any one of the

preceding claims) which is:
(a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or
(b) in a pharmaceutical excipient, a unit dosage form or in a form suitable for local or systemic administration.

13. An antibody (or antibody derivative) specific for the factor (or homologue, derivative, allelic form, species variant, mutein or equivalent) as defined in any one of the preceding claims.

14. The antibody of claim 13 which is:
(a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or
(b) in a pharmaceutical excipient, a unit dosage form or in a form suitable for local or systemic administration.

15. Isolated RP-factor receptor or convertase.

16. The receptor/convertase of claim 15 which is derived from a source as defined in any one of claims 4-6.

17. A homologue, derivative, allelic form, species variant, mutein or equivalent of the receptor/convertase of claim 15 or 16.

18. Recombinant RP-factor receptor/convertase, wherein the receptor/convertase is for example as defined in any one of claims 15-17.

19. A pharmaceutical composition (e.g. a vaccine) comprising the receptor/convertase (or homologue, derivative, allelic form, species variant, mutein or equivalent) as defined in any one of claims 15-18.

20. The receptor/convertase (or homologue, derivative, allelic form, species variant, mutein or equivalent) or pharmaceutical composition as defined in any one of claims 15-19 which is:
(a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or
(b) in a pharmaceutical excipient, a unit dosage form or in a form suitable for local or systemic administration.

21. An antibody (or antibody derivative) specific for the receptor (or homologue, derivative, allelic form, species variant, mutein or equivalent) as defined in any one of claims 15-20.

22. The antibody of claim 21 which is:
(a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or
(b) in a pharmaceutical excipient, a unit dosage form or in a form suitable for local or systemic administration.

23. An RP-factor antagonist or inhibitor.

24. The antagonist or inhibitor of claim 23 which comprises:
(a) the antibody of claim 13, 14, 21 or 22; and/or
(b) the receptor of claims 15-20; and/or
(c) an RP-factor mutein which comprises an altered RP-factor specificity-determining domain or which lacks a functional signalling domain.

25. The antagonist or inhibitor of claim 23 or 24 which is:
(a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or
(b) in a pharmaceutical excipient, a unit dosage form or in a form suitable for local or systemic administration.

26. An RP-factor agonist, activator or mimetic.

27. The agonist, activator or mimetic of claim 26 which comprises:
(a) the antibody of claim 21 or 22; and/or
(b) an RP-factor mutein comprising (or consisting of) an RP-factor specificity-determining domain; and/or
(c) an RP-factor mutein comprising (or consisting of) an RP-factor signalling domain; and/or
(d) an RP-factor convertase; and/or
(e) operably coupled combinations of any of (a)-(d).

28. The agonist, activator or mimetic of claim 27 which is:
(a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or
(b) in a pharmaceutical excipient, a unit dosage form, in a form suitable for local or systemic administration or in admixture with an antibiotic.

29. The agonist, activator or mimetic of claim 28 which is for use in adjunctive therapy (for example in combination with an antibiotic).

30. Isolated nucleic acid encoding the RP-factor (or homologue, derivative, allelic form, species variant, mutein or equivalent thereof) or RP-factor receptor as defined in the preceding claims.

31. A vector (e.g. an expression vector) comprising the nucleic acid of claim 30.

32. A host cell comprising the vector of claim 31.

33. A culture or transport medium comprising an RP-factor (e.g. the factor (or homologue, derivative, allelic form, species variant, mutein or equivalent) as defined in the preceding claims), for example comprising a culture supernatant containing an RP-factor.

34. A nucleic acid probe comprising nucleic acid complementary to the nucleic acid of claim 30.

35. A diagnostic kit comprising an RP-factor (or homologue, derivative, allelic form, species variant, mutein or equivalent), receptor, antibody, probe, culture supernatant or culture medium as defined in any one of the preceding claims.

36. Antisense DNA corresponding to the nucleic acid of claim 30.

37. A process for producing an antimicrobial drug comprising the steps of:
(a) providing an RP-factor receptor;
(b) providing candidate drugs;
(c) screening the candidate drugs by contacting the RP-factor receptor with one of the candidate drugs and determining the affinity of the candidate drug for the RP-factor receptor, wherein the affinity is an index of antimicrobial activity, and optionally
(d) synthesising or purifying a drug having antimicrobial activity on the basis of the identity of the candidate drug screened in step (c).

38. A process for producing an antimicrobial drug comprising the steps of:
(a) providing an RP-factor receptor;
(b) providing a candidate drug;
(c) providing an RP-factor;
(d) screening the candidate drugs by contacting the RP-factor receptor with one of the candidate drugs in the presence of the RP-factor, and then determining the ability of the candidate drug to compete non-productively with the RP-factor for binding to the RP-factor receptor, wherein the competitive binding ability is an index of antimicrobial activity, and optionally
(e) synthesising or purifying a drug having antimicrobial activity on the basis of the identity of the candidate drug screened in step (d).

39. An antimicrobial drug produced by (or obtainable by) the process of claim 37 or 38, or a derivative thereof.

40. A method for determining the microbiological quality of a product (e.g. a foodstuff, pharmaceutical preparation or medical product) comprising the step of contacting a sample of the product with an RP-factor (for example, an RP-factor as defined in the preceding claims).

41. A method of culturing bacterial (e.g. mycobacterial) cells, comprising the step of incubating the cells in a culture medium comprising an RP-factor (for example, an RP-factor as defined in the preceding claims).

42. An *ex vivo* method of diagnosis, comprising the step of contacting a biological sample with an RP-factor (for example, an RP-factor as defined in the preceding claims).

43. The method of claim 42 wherein the biological sample is incubated with a culture or transport medium as defined in claim 33.

44. A method of:
(a) stimulating the growth of a microorganism; and/or
(b) resuscitating a dormant, moribund or latent microorganism;
comprising the step of contacting the microorganism with an RP-factor (for example, an RP-factor as defined in the preceding claims).

45. A process for producing the RP-factor or RP-factor receptor of the invention comprising the steps of:

(a) culturing the host cell of claim 32, and

(b) purifying the factor or receptor from the cultured host cells (e.g. from a culture supernatant or cell fraction).

46. A process for producing the RP-factor or receptor of the invention comprising the steps of:

(a) probing a gene library with a nucleic acid probe which is selectively hybridizable with the nucleic acid of claim 30 to produce a signal which identifies a gene that selectively hybridises to the probe;

(b) expressing the gene identified in step (a) (for example by cloning into a host cell, e.g. according to a process as defined in claim 45) to produce the factor or receptor.

47. An RP-factor or receptor obtainable by the process of claim 45 or 46.

48. A process for producing a library of biomolecules comprising the steps of:
(a) providing a sample (e.g. a soil, marine, food, freshwater, tissue or organism-derived);
(b) incubating the sample in a culture medium comprising an RP-factor (for example, an RP-factor as defined in the preceding claims or a culture supernatant comprising an RP-factor) to produce a microbial culture;
(c) isolating microorganisms from the culture of step (b).

49. The process of claim 48 further comprising the step of screening the isolated microorganisms for those which elaborate one or more biomolecules of interest (for example a metabolite, enzyme, antibiotic (e.g. antiviral, antibacterial or antifungal agent) or toxin).

50. A biomolecule produced by (or obtainable by) the process of claim 48 or 49, or a derivative thereof.

51. A process for producing a library of microorganisms (e.g. bacteria) comprising the steps of:
(a) providing a sample (e.g. a soil, marine, food, freshwater, tissue or organism-derived sample);

(b) incubating the sample in a culture medium comprising an RP-factor (for example, an RP-factor as defined in the preceding claims or a culture supernatant comprising an RP-factor) to produce a microbial culture;
(c) isolating microorganisms from the culture of step (b); and optionally
(d) culturing and/or mutagenising the microorganism.

52. A microorganism produced by (or obtainable by) the process of claim 51, or a derivative (e.g. mutant) thereof.

53. Use of a culture supernatant (or fraction or extract thereof) containing an RP-factor for:
(a) diagnosis, prophylaxis or therapy; or
(b) producing a library of microorganisms (e.g. according to the method of claim 51); or
(c) producing a library of biomolecules (e.g. according to the method of claim 48); and/or
(d) resuscitating a dormant, moribund or latent pathogen (e.g. according to the method of claim 44(b)).

54. A culture supernatant (or fraction or extract thereof) containing an RP-factor for use in therapy, prophylaxis or diagnosis.

55. An *ex vivo* method of diagnosis comprising the step of incubating a sample with a culture supernatant (or fraction or extract thereof) containing an RP-factor (or an RP-factor as defined in any one of the preceding claims) at a concentration sufficient to promote the recovery of microrganisms from the sample by culture.

56. The method of claim 55 wherein the sample:
(i) is from an accessible body site, for example a mucous membrane of the vagina, anus, nose, urethra, cervix, skin, conjunctiva, mouth or throat; and/or
(ii) comprises a fluid or semi-solid (for example a bodily fluid or semi-solid, e.g. discharge, vomit, secretion, excreta, sputum or blood); and/or
(iii) comprises a solid (e.g. stool, tissue, food or biopsy sample); and/or
(iv) comprises a culture (e.g. a microbiological culture).

57. A live vaccine comprising an attenuated microbe, which microbe bears a mutation in a gene encoding (or regulating the expression or activity of) one or more RP-factors.

58. The vaccine of claim 57 wherein the microbe selected from any of: an actinomycete, mycobacterium (for example *M. tuberculosis, M. leprae, M. bovis* (e.g. *M. bovis* BCG) and *M. avium*), *Corynebacterium* spp. (e.g. *Corynebacterium diphtheriae*), *Tropheryma whippelii, Nocardia* spp. (e.g. *Nocardia asteroides* and *Nocardia brasiliensis*), *Streptomyces* spp. (e.g. *Streptomyces griseus, Streptomyces paraguayensis* and *Streptomyces somaliensis*), *Actinomadura* spp., *Nocardiopsis* spp., *Rhodococcus* spp., *Gordona* spp., *Tsukamurella* spp. and *Oerskovia* spp., a pathogenic high G+C Gram-positive bacterium and a pathogenic low G+C Gram-positive bacterium (for example *Streptococcus* spp., *Staphylococcus* spp., *Listeria* spp., *Bacillus* spp., *Clostridium* spp.

and *Lactobacillus* spp.).

59. The vaccine of claim 57 or claim 58 wherein the mutation is selected from any of: frameshift, deletion, insertion and/or substitution mutations.

60. The vaccine of any one of claims 57-59 wherein the mutation:

(a) comprises a null mutation (e.g. a non-reverting null mutation); and/or

(b) prevents growth of the microbe; and/or

(c) results in the expression of a mutant RP-factor having altered specificity (e.g. in which the specificity-determining domain has been mutated or modified) and/or which lacks a functional signalling domain.

add A1 add A1

FIG. 1A

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MtubZ94752      mlrlvvgalllvlafaggyavaacktvtltvdgtamrvttmksrvidive      50

MtubZ94752      engfsvddrddlypaagvqvhdadtivlrrsrplqislqghdakqvwtta     100

MtubZ94752      stvdealaqlamtdtapaaasrasrvplsgmalpvvsaktvqlndgglvr     150

MtubZ94752      tvhlpapnvagllsaagvpllqsdhvvpaatapivegmqiqvtrnrikkv     200
MtubMTV008      ----------------mpvgwlwrartakgttlknarttliaaaiagt      32

Mlepl04666      ----------------------------------------msesyrkl       8
MtubMTV043      ----------------------------------------msgrhrkpt       9
MtubZ94752      terlplppnarrvedpemnmsrevvedpgvpgtqdvtfavaevngvetgr     250
MlutZ96935      ----------------------------------------mtlfttsat       9
MlepL01095      ------------------------------------mpgemldvrklc      12
MtubU38939      ----------mhplpadhgrsrcnrhpisplsliqnisatsqdmssmt       38
MtubZ81368      --------------------------------mtpgllttagaqrprdrca      19
MtubMTV008      lvttspagianaddagldpnaaagpdavgfdpnlppapdaapvdtppape      82

Scoeli6C12$     ---irtaavtlvaatalgatgeavaapsaplrtDWDAIAACESSGNWQAN      25
Mlepl04666      ttssiivakitftgamldgsialagqaspatdsEWDQVARCESGGNWSIN      58
MtubMTV043      tsnvsvakiaftgavlggggiamaaqataatdgEWDQVARCESGGNWSIN      59
MtubZ94752      lpvanvvvtpaheavvrvgtkpgtevppvidgsIWDAIAGCEAGGNWAIN     300
MlutZ96935      rsrratasivagmtlagaaavgfsapaqaatvdTWDRLAECESNGTWDIN      59
MlepL01095      klfvksavvsgivtasmalststgmanavprePNWDAVAQCESGRNWRAN      62
MtubU38939      riakpliksamaaglvtasmslstavahagpsPNWDAVAQCESGGNWAAN      88
MtubZ81368      rivctvfietavvatmfvallglstisskaddIDWDAIAQCESGGNWAAN      69
MtubMTV008      dagfdpnlppplapdflsppaeeappvpvaysVNWDAIAQCESGGNWSIN     132
                                                 ***.* *******  *

Scoeli6C12$     TGNGYYGGLQFARSSWIAAGGLKYAPRADLATRGEQIAVAERLARLQGMS      75
Mlepl04666      TGNGYLGGLQFSQGTWASHGGGEYAPSAQLATREQQIAVAERVLATQGSG     108
MtubMTV043      TGNGYLGGLQFTQSTWAAHGGGEFAPSAQLASREQQIAVGERVLATQGRG     109
MtubZ94752      TGNGYYGGVQFDQGTWEANGGLRYAPRADLATREEQIAVAEVTRLRQGWG     350
MlutZ96935      TGNGFYGGVQFTLSSWQAVGGEG---YPHQASKAEQIKRAEILQDLQGWG     106
MlepL01095      TGNGFYGGLQFKPTIWARYGGVG---NPAGASREQQITVANRVLADQGLD     109
MtubU38939      TGNGKYGGLQFKPATWAAFGGVG---NPAAASREQQIAVANRVLAEQGLD     135
MtubZ81368      TGNGLYGGLQISQATWDSNGGVG---SPAAASPQQQIEVADNIMKTQGPG     116
MtubMTV008      TGNGYYGGLQFTAGTWRANGGSG---SAANASREEQIRVAENVLRSQGIR     179
                ****.******   .* . **          *.***** *** ..  **

Scoeli6C12$     AW----------------------------------------------      78
Mlepl04666      AWPACGHGLSGPSLQEVLPAG--MGAPw----INGAPAPLAPPPPAEPAP     152
MtubMTV043      AWPVCGRGLSNATPREVLPASaaMDAPldaaaVNGEPAPLA-PPPADPAP     158
MtubZ94752      AWPVCAaragar-----------------------------------------     362
MlutZ96935      AWPLCSQKLgltqadadagdvdateaapvavertatvqrqsaadeaaaeq     156
MlepL01095      AWPKCGAASDLPITLWSHPAQGVKQIINDIIqmgdttlaaialngl----     155
MtubU38939      AWPTCGAASGLPIALWSKPAQGIKQIINEIIwagiqasipr---------     176
MtubZ81368      AWPKCSscsqgdaplgslthiltflaaetggcsgsrdd------------     154
MtubMTV008      AWPVCGrrg---------------------------------------     188
                *** *.
```

FIG. 1A (CONT.)

```
Mlepl04666      pqppadnf----------------------PPTPGDVPSPLarp-----   174
MtubMTV043      pvelaandlpaplgeplpaapadpappadlaPPAPADVAPPVelavndlp  208
MlutZ96935      aaaaeqavvaeaetivvksgdslwtlaneyeveggwtalyeankgavsda  206

MtubMTV043      aplgeplpaapadpappadlappapadlappapadlappapadlappvel  258
MlutZ96935      aviyvgqelvlpqa-----------------------------------  220

MtubMTV043      avndlpaplgeplpaapaelappadlapasadlappapadlappapaela  308

MtubMTV043      ppapadlappaavneqtapgdqpatapggpvglatdlelpepdpqpadap  358

MtubMTV043      ppgdvteapaetpqvsniaytkklwqairaqdvcgndaldslaqpyvig-  407
```

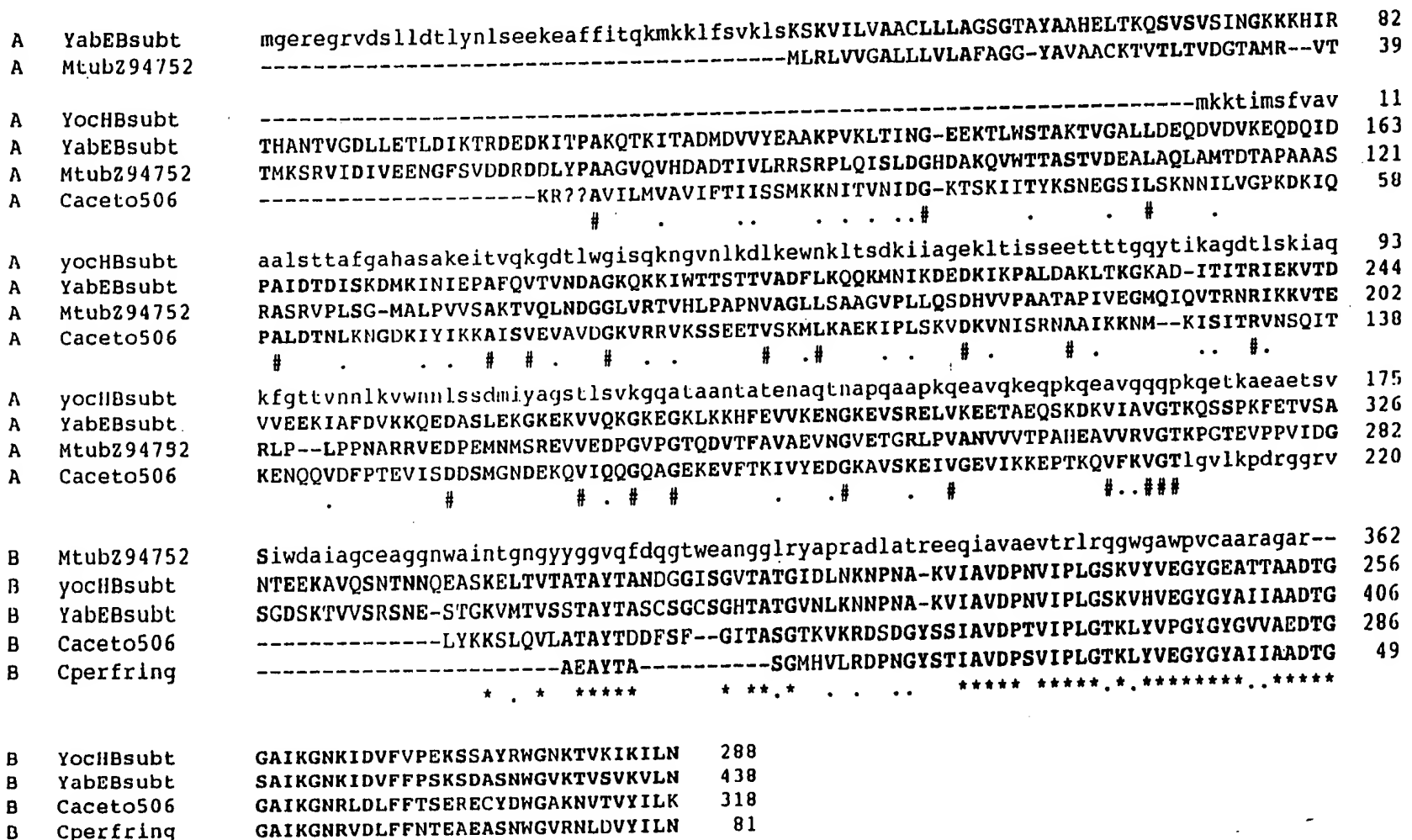

FIG. 1B

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```
RPF        TIVVKSGDSLWTLANE-YEVEGGWTALYEANKGAVS---------DAAVIYVGQELVLPQA
g149657    TIKVKSGDSLWKLSRQ-YDTT--ISALKSENKL------------KSTVLYVGQSLKVPES
g2226145   TIKVKSGDSLWKLAQT-YNTS--VAALTSANNL------------STTVLSIGQTLTIP--
g2226145   TYTVKSGDSLWVIAQK-FNVT--AQQIREKNNL------------KTDVLQVGQKLVI---
g2226145   KYTVKSGDSLWKIANN-INLT--VQQIRNINNL------------KSDVLYVGQVLKL---
g266725    TYTVKSGDTIWALSSK-YGTS--VQNIMSWNNL------------SSSSIYVGQVLAVKQ-
g80581     THAVKSGDTIWALSVK-YGVS--VQDIMSWNNL------------SSSSIYVGQKLAIKQ-
g2707292   SVKVKSGDTLWALSVK-YKTS--IAQLKSWNHL------------SSDTIYIGQNLIVSQS
g755216    TYTVKSGDTLWGISQR-YGIS--VAQIQSANNL------------KSTIIYIGQKLLL---
g1722873   TYTVKKGDTLWDIAGRFYGNSTQWRKIWNANKTAMIKRSKRNIRQPGHWIFPGQKLKIPQ-
g1176755   TYTVKKGDTLWDLAGKFYGDSTKWRKIWKVNKKAMIKRSKRNIRQPGHWIFPGQKLKIPQ-
           .  **.** .*       .     .    *.                .  ** *
```

FIG. 1C

```
1 msgrhrkpttsnvsvakiaftgavlggggiamaaqataatdgewdqvarcesggnwsintgngylgg
  lqftqstwaahgggefapsaqlasreqqiavgervlatqgrgawpvcgrglsnatprevlpasaamd
  apldaaavngepaplapppadp 156

157 appvelaandlpaplgeplpaapadpappadlappapadv 196
197 appvelavndlpaplgeplpaapadpappadlappapadlappapadlappapadl 252
253 appvelavndlpaplgeplpaapaelappadlap-asadlappapadlappapaelappapadlappa
320 ------avne 323

324 qtapgdqpatapggpvglatdlelpepdpqpadapppgdvteapaetpqvsniaytkklwqaira
389 qdvcgndaldslaqpyvig* 407
```

Motif	sequence	
A	157 appvelaandl	167
B'	168 paplgeplpaapad	181
C	182 pappadl	188
D	189 appapadv	196
A	197 appvelavndl	207
B'	208 paplgeplpaapad	221
C	222 pappadl	228
D	229 appapadl	236
D	237 appapadl	244
D	245 appapadl	252
A	253 appvelavndl	263
B	264 paplgeplpaapael	278
'C	279 appadl	284
D*	285 apasadl	291
D	292 appapadl	299
D	300 appapael	307
D	308 appapadl	315
D'	316 appa	319
'A'	320 avne	323

A = appvela[av]ndl

B = paplgeplpaapa[de]l

C = pappadl

D = appapa[de][lv]

FIG. 1D

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Lmonocytog.. 72
MlutFactor 62
Lmonocytog.. 144
MlutFactor 125
Lmonocytog.. 216
MlutFactor 184
Lmonocytog.. 283
MlutFactor 220
Lmonocytog.. 355
MlutFactor 220
Lmonocytog.. 427
MlutFactor 220
Lmonocytog.. 478
MlutFactor 220

FIG. 1E

```
  1 accaaggagaaggacgaccccggtgtgcctcggccgccgatcagcgaggactcgccatgg 60
 61 acaccatgactctcttcaccacttccgccacccgctcccgccgtgccaccgcctcgatcg 120
        M  T  L  F  T  T  S  A  T  R  S  R  R  A  T  A  S  I  V
                                                              g
121 tcgcgggcatgaccctcgccggcgccgccgccgtgggcttctccgccccggcccaggccg 180
      A  G  M  T  L  A  G  A  A  A  V  G  F  S  A  P  A  Q  A  A
                                                              A
                            oligo A1>>>
    csacsgtsgacacstgggaccgsctsgcsgag
181 ccaccgtggacacctgggaccgcctcgccgagtgcgagtccaacggcacctgggacatca 240
      T  V  D  T  W  D  R  L  A  E  C  E  S  N  G  T  W  D  I  N
      T  V  D  T  W  D  R  L  A  E  E  x  S  N  G  T  x  D
           <<< oligo G2                        oligo G1>>>
           gttgccgaagatgccgcc      agttcaccctgtcctcctg
241 acaccggcaacggcttctacggcggcgtgcagttcaccctgtcctcctggcaggccgtcg 300
      T  G  N  G  F  Y  G  G  V  Q  F  T  L  S  S  W  Q  A  V  G
                                                              V  G
      <<< oligo A2
      ccictycciatrggigtrgtycg
301 gcggcgaaggctacccgcaccaggcctcgaaggccgagcagatcaagcgcgccgagatcc 360
      G  E  G  Y  P  H  Q  A  S  K  A  E  Q  I  K  R  A  E  I  L
      G  E  G  Y  P  H  Q  A  S  K
361 tccaggacctgcagggctggggcgcgtggccgctgtgctcgcagaagctgggcctgaccc 420
      Q  D  L  Q  G  W  G  A  W  P  L  C  S  Q  K  L  G  L  T  Q
421 aggctgacgcggacgccggtgacgtggacgccaccgaggccgccccggtcgccgtggagc 480
      A  D  A  D  A  G  D  V  D  A  T  E  A  A  P  V  A  V  E  R
481 gcacggccaccgtgcagcgccagtccgccgcggacgaggctgccgccgagcaggccgctg 540
      T  A  T  V  Q  R  Q  S  A  A  D  E  A  A  A  E  Q  A  A  A
541 ccgcggagcaggccgtcgtcgccgaggccgagaccatcgtcgtcaagtccggtgactccc 600
      A  E  Q  A  V  V  A  E  A  E  T  I  V  V  K  S  G  D  S  L
601 tctggacgctcgccaacgagtacgaggtggagggtggctggaccgccctctacgaggcca 660
      W  T  L  A  N  E  Y  E  V  E  G  G  W  T  A  L  Y  E  A  N
661 acaagggcgccgtctccgacgccgccgtgatctacgtcggccaggagctcgtcctgccgc 720
      K  G  A  V  S  D  A  A  V  I  Y  V  G  Q  E  L  V  L  P  Q
721 aggcctgagacgcctgaccggcccccccggaccggtacc 758
      A  *

  1   ATVDTWDRLA ECESNGTWDI NTGNGFYGGV QFTLSSWQAV GGEGYPHQAS KAEQIKRAEI     60
 61   LQDLQGWGAW PLCSQKLGLT QADADAGDVD ATEAAPVAVE RTATVQRQSA ADEAAAEQAA    120
121   AAEQAVVAEA ETIVVKSGDS LWTLANEYEV EGGWTALYEA NKGAVSDAAV IYVGQELVLP QA 182
```

FIG. 2A

```
ggatccgcaccgccgcggtaaccctggtcgccgcgaccgcactcggggcgaccggcgaag  60
  I  R  T  A  A  V  T  L  V  A  A  T  A  L  G  A  T  G  E  A

cggtggccgcgccctcggcgcccctgcgcaccgactgggacgccatcgccgcgtgcgagt 120
  V  A  A  P  S  A  P  L  R  T  D  W  D  A  I  A  A  C  E  S

ccagcggcaactggcaggcgaacaccggcaacggctactacggcggcctgcagttcgcac 180
  S  G  N  W  Q  A  N  T  G  N  G  Y  Y  G  G  L  Q  F  A  R

ggtccagctggatcgccgccggcggcctcaagtacgccccgcgcgcggacctcgccaccc 240
  S  S  W  I  A  A  G  G  L  K  Y  A  P  R  A  D  L  A  T  R

gcggcgagcagatcgccgtggcggaacgcctcgcccgtctgcaggggatgtccgcctgg  299
  G  E  Q  I  A  V  A  E  R  L  A  R  L  Q  G  M  S  A  W
```

FIG. 2B

FIG. 3

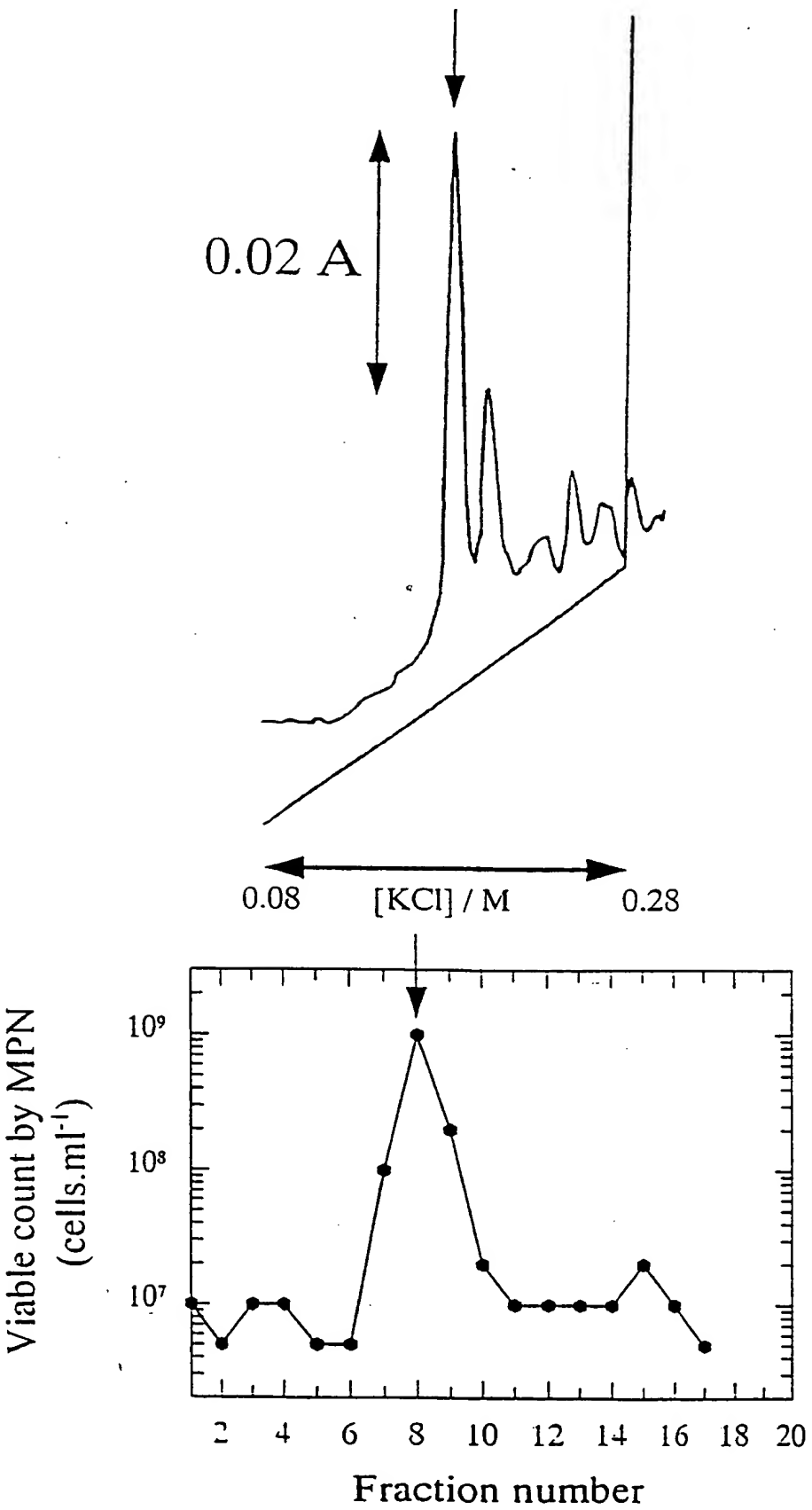
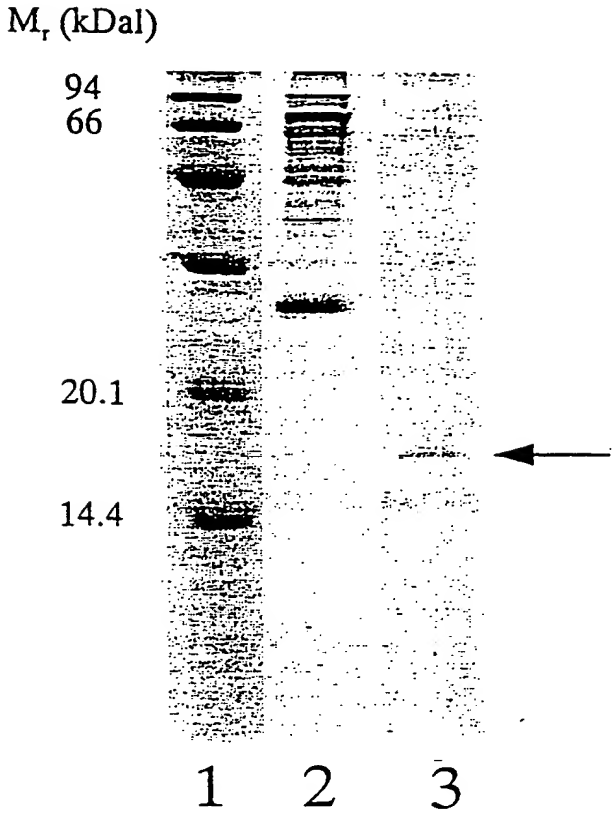

A
0.02 A
B
0.08
[KCl] / M
0.28
Viable count by MPN
(cells.ml-1)
10⁹
10⁸
10⁷
2
4
6
8
10
12
14
16
18
20
Fraction number

FIG. 3

C.


M$_r$ (kDal)
94
66
20.1
14.4
1
2
3

D.

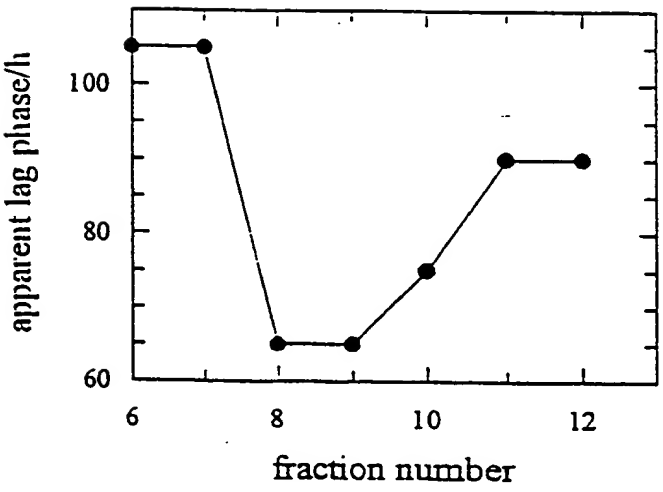
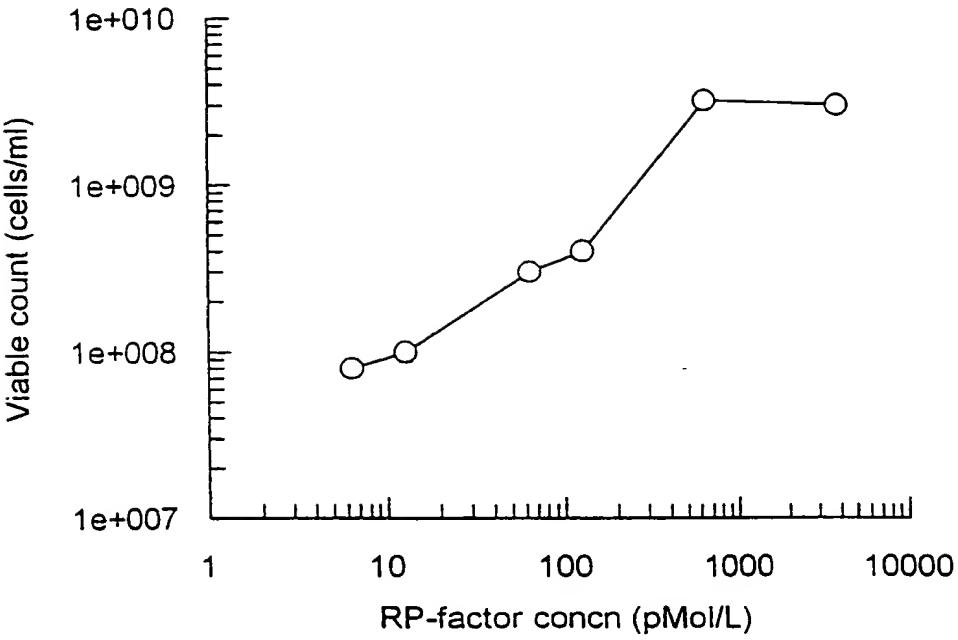

apparent lag phase/h
100
80
60
6
8
10
12
fraction number

FIG. 4

A


1e+010
1e+009
1e+008
1e+007
Viable count (cells/ml)
1
10
100
1000
10000
RP-factor concn (pMol/L)

B

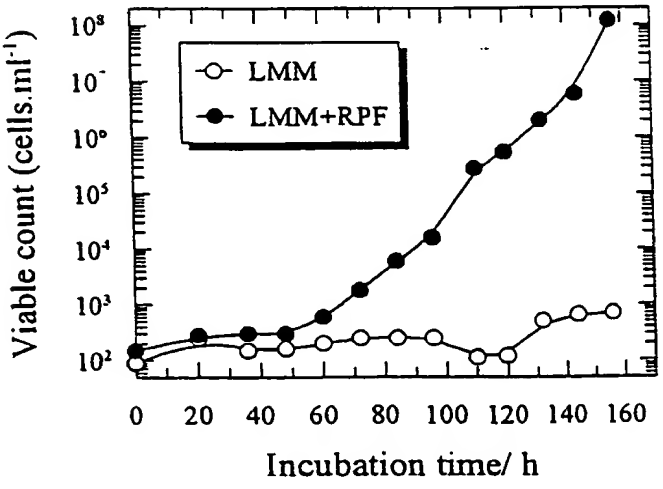
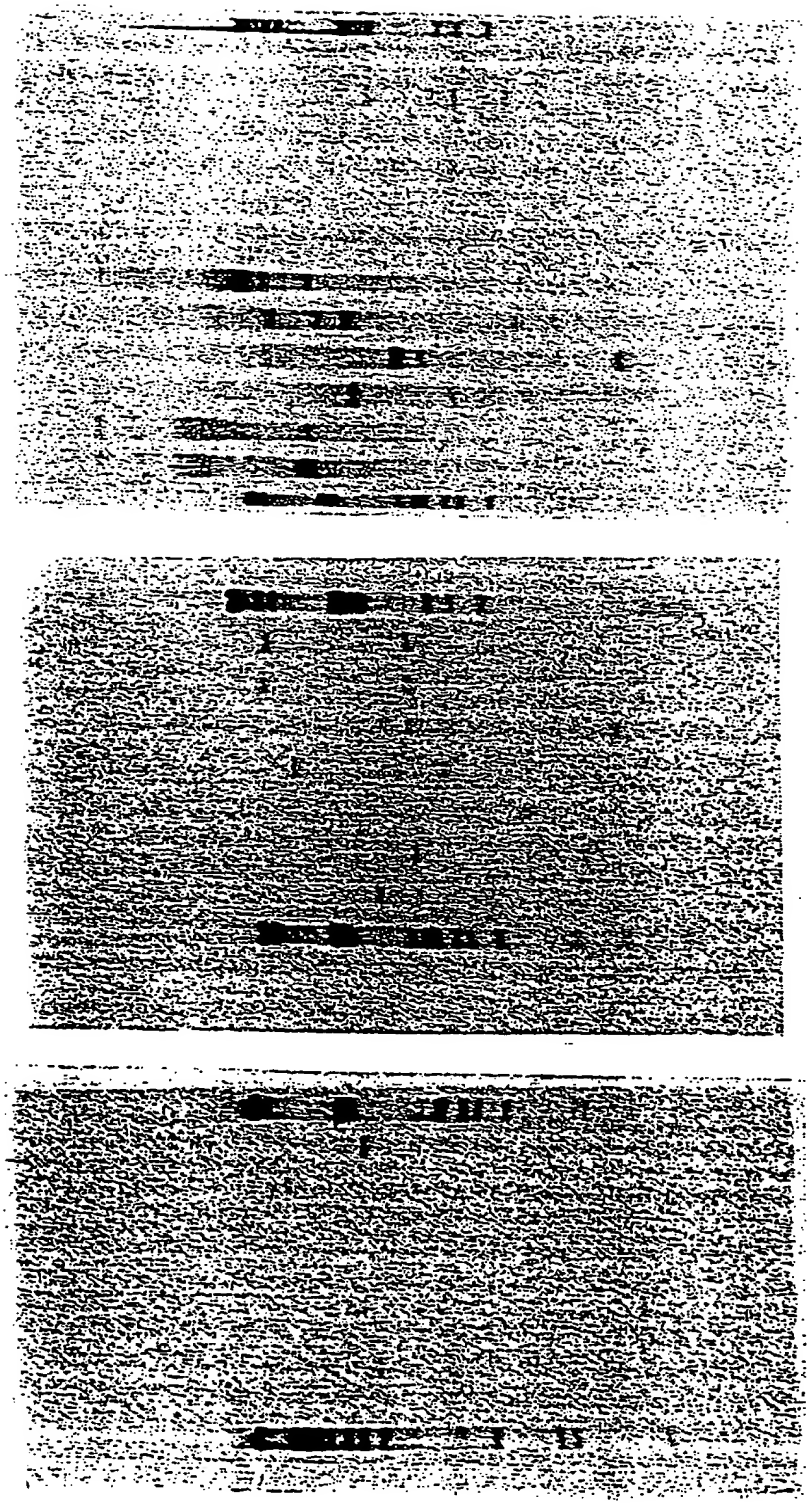

LMM
LMM+RPF
Viable count (cells.ml⁻¹)
0
20
40
60
80
100
120
140
160
Incubation time/ h

FIG.5



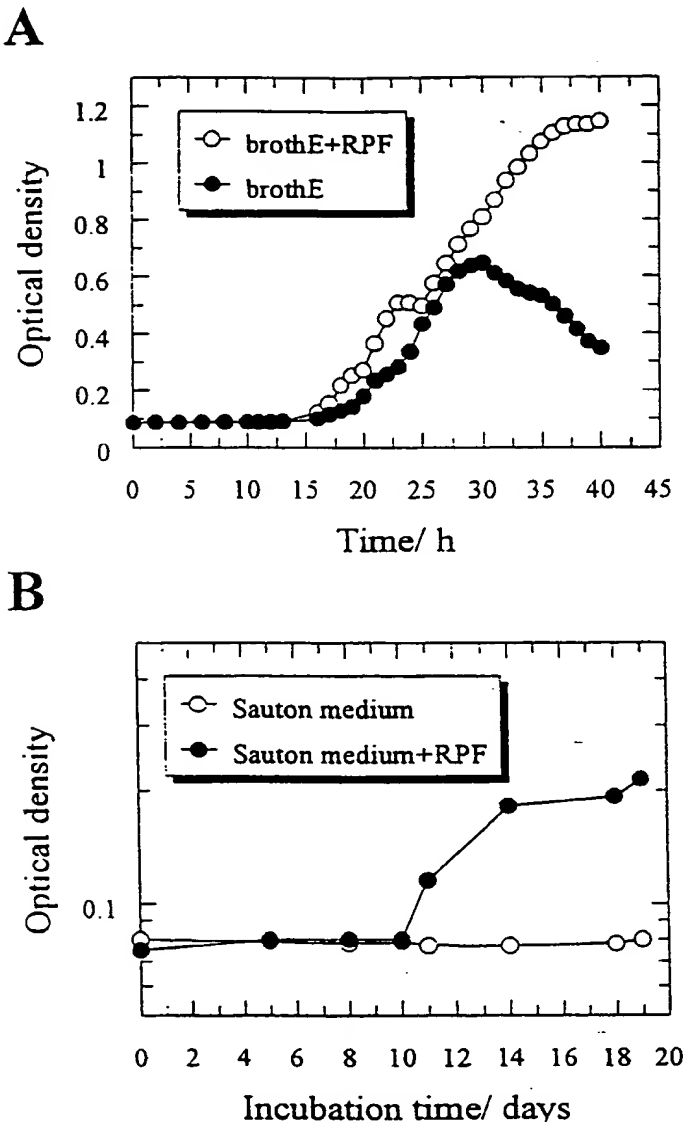

A
Optical density
1.2
1
0.8
0.6
0.4
0.2
0
brothE+RPF
brothE
0 5 10 15 20 25 30 35 40 45
Time/ h
B
Optical density
0.1
Sauton medium
Sauton medium+RPF
0 2 4 6 8 10 12 14 16 18 20
Incubation time/ days

FIG. 6

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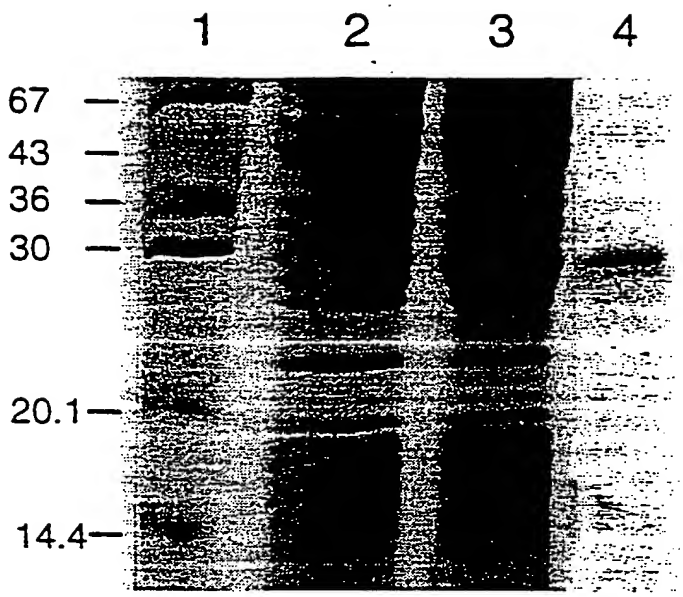
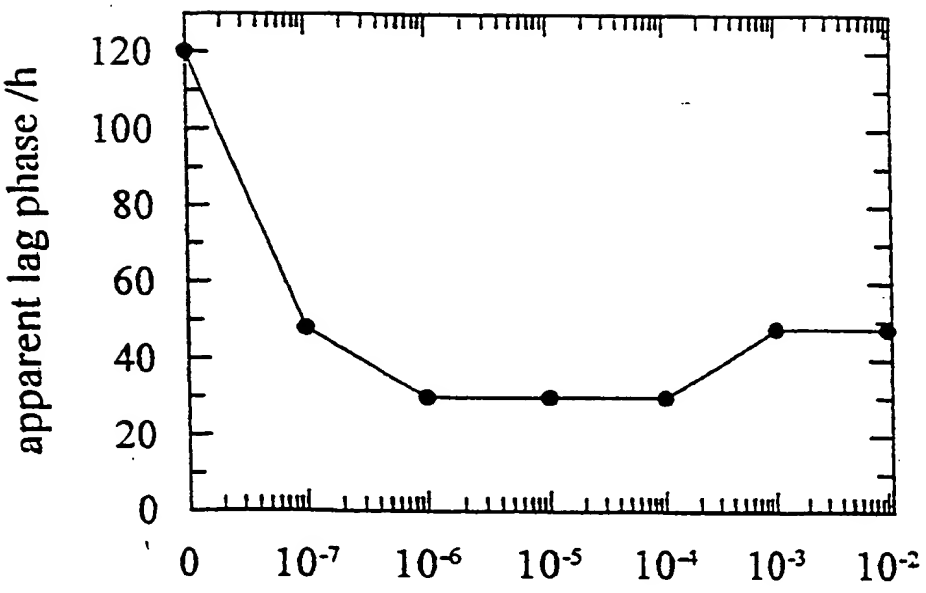
FIG. 7

A


1
2
3
4
67
43
36
30
20.1
14.4

B


apparent lag phase /h
120
100
80
60
40
20
0
0
10-7
10-6
10-5
10-4
10-3
10-2
RP-factor dilution

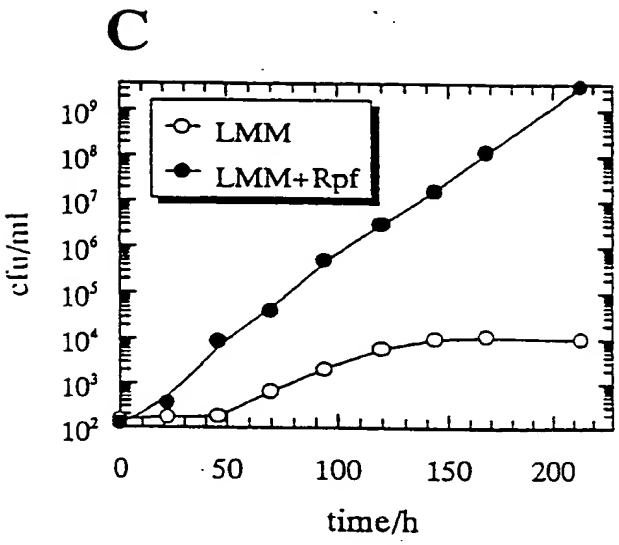


FIG. 7

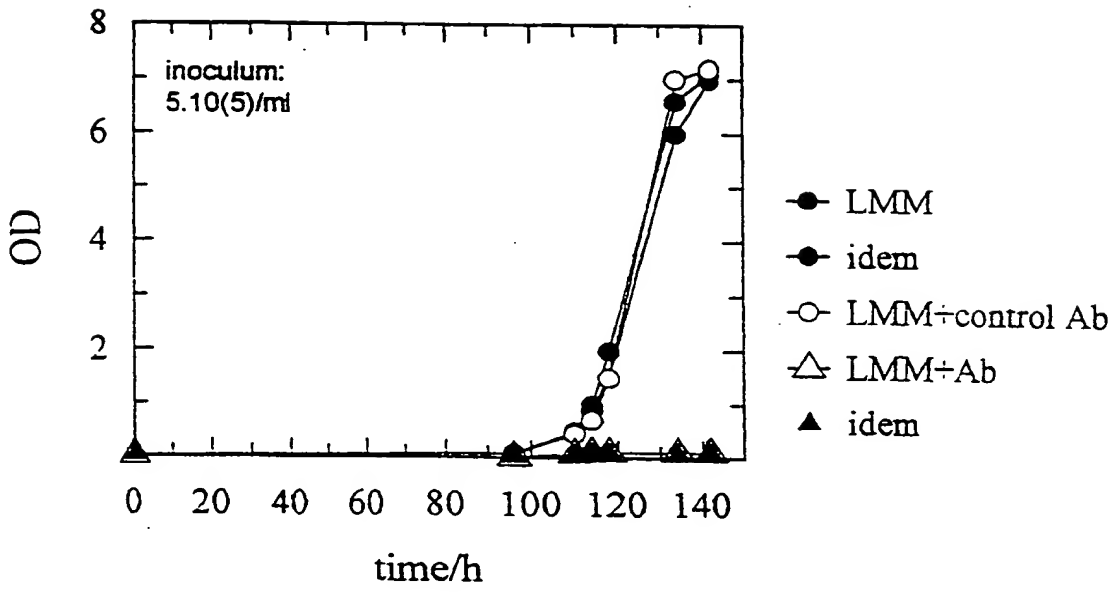


FIG. 8A

FIG. 8 B

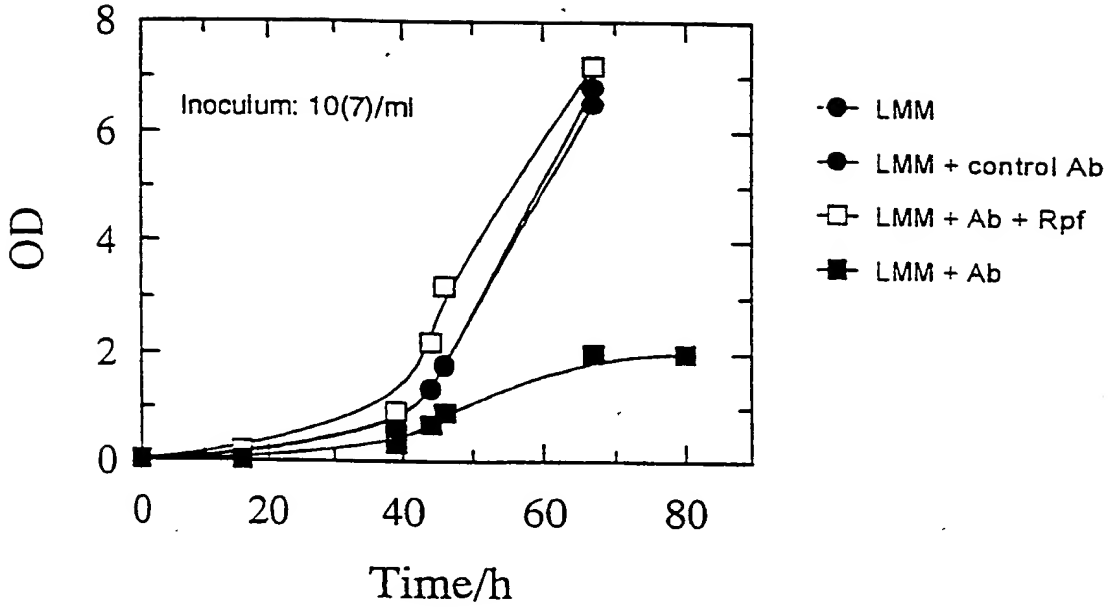
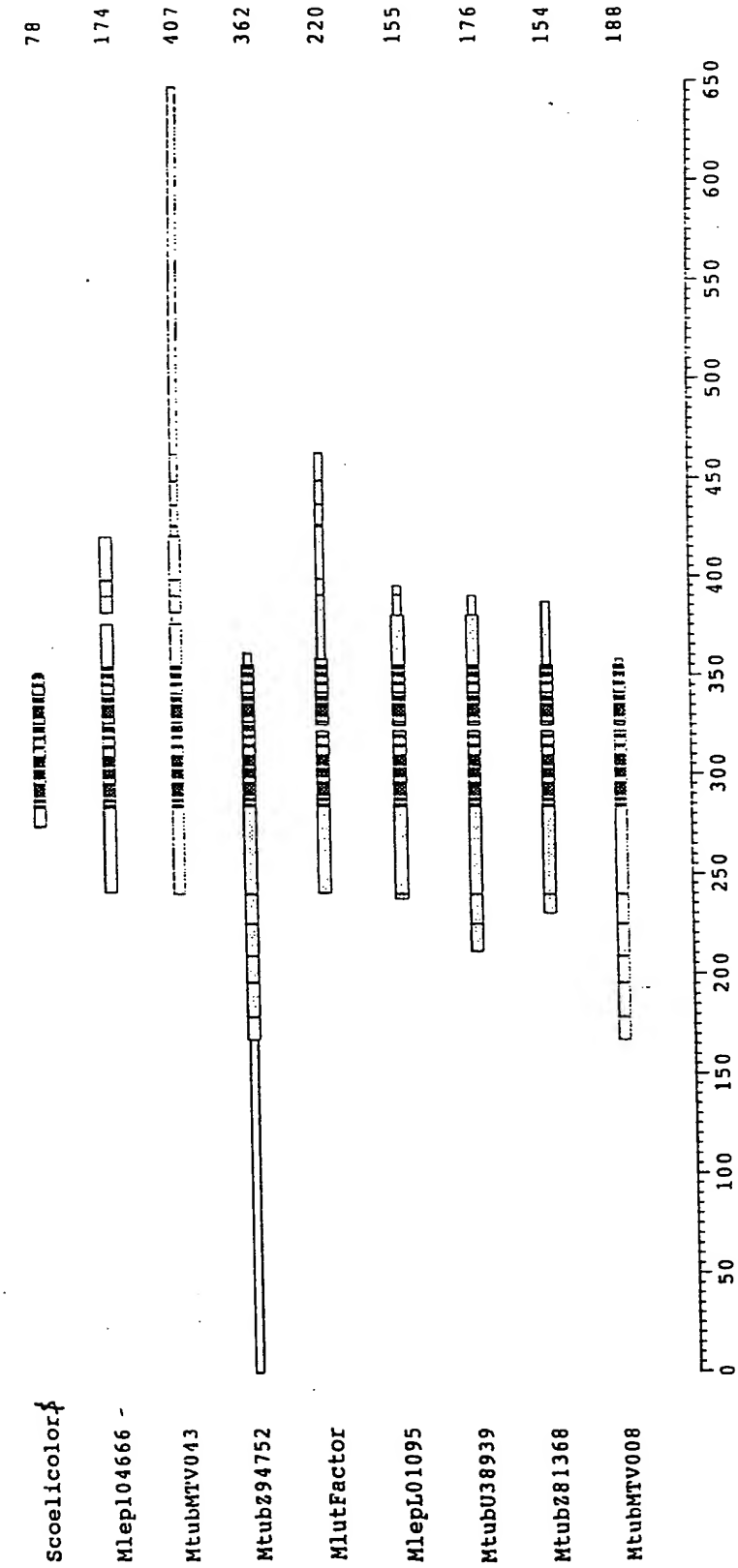


FIG. 9A


78
174
407
362
220
155
176
154
188
Scoelicolor4
Mlep104666 -
MtubMTV043
Mtub294752
MlutFactor
MlepL01095
MtubU38939
Mtub281368
MtubMTV008
0
50
100
150
200
250
300
350
400
450
500
550
600
650
sequence position

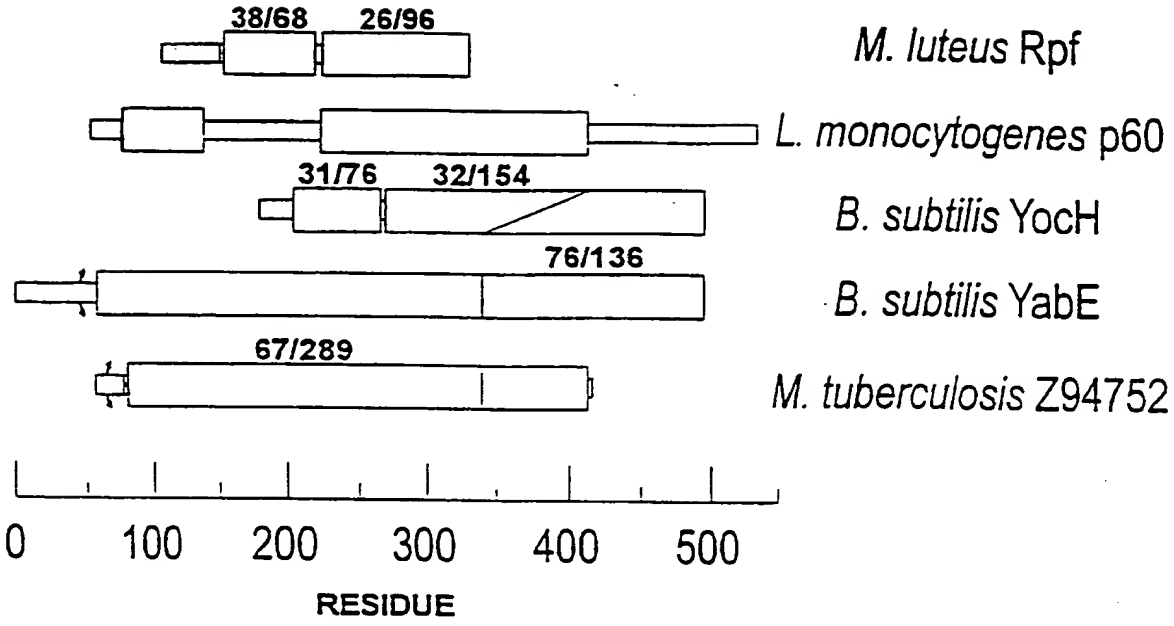


FIG. 9B

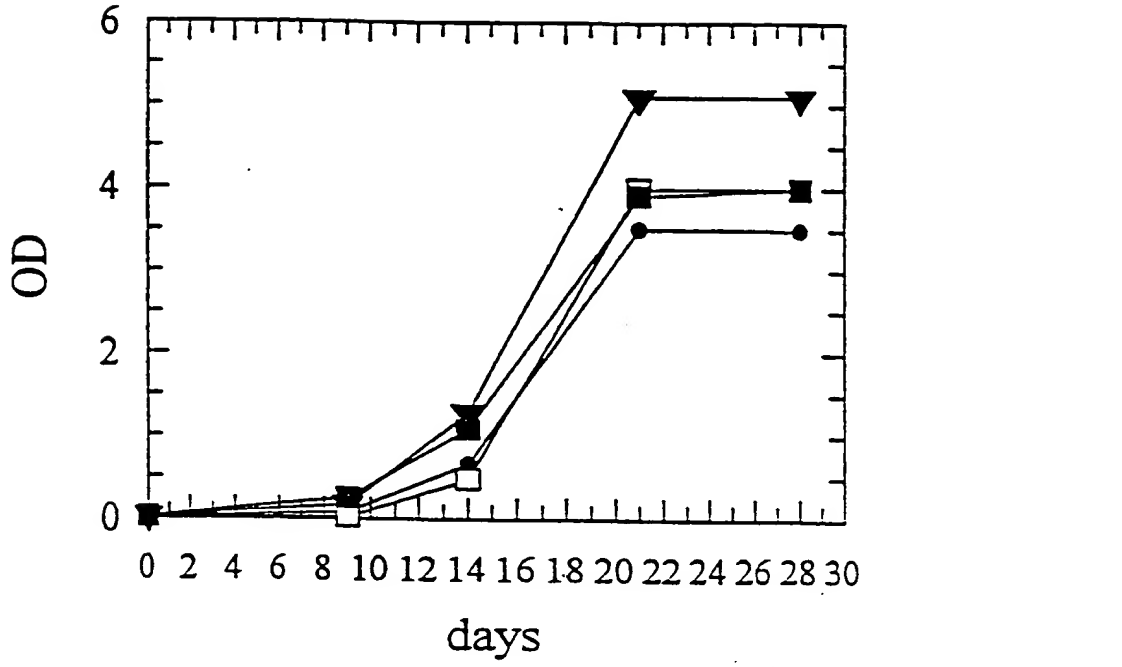


FIG. 10

INTERNATIONAL SEARCH REPORT

[redacted] Application No
PCT/[redacted]98/01619

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/31 C07K14/195 C07K14/315 C07K14/31 C07K14/32
C07K14/33 C07K14/335 C07K14/305 C07K14/35 C07K14/36
C07K14/34 C12N1/38 A61K39/02 A61K39/05 A61K39/07

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A61K G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	MUKAMOLOVA G V ET AL: "A bacterial cytokine." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1998 JUL 21) 95 (15) 8916-21. JOURNAL CODE: PV3. ISSN: 0027-8424., XP002080580 United States see the whole document --- -/--	1-10, 15-18, 44,47

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 October 1998	04/11/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Espen, J

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 98/01619

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/08 A61K39/085 C07K16/12 G01N33/50 C12Q1/68
C12N15/11

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VOTYAKOVA T V ET AL: "Influence of viable cells on the resuscitation of dormant cells in Micrococcus luteus cultures held in an extended stationary phase: The population effect." APPLIED AND ENVIRONMENTAL MICROBIOLOGY 60 (9). 1994. 3284-3291. ISSN: 0099-2240, XP002080581	33
Y	see the whole document	1-10, 15-18, 44,47

-/--

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 October 1998	

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Espen, J

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KAPRELYANTS A S ET AL: "Estimation of dormant Micrococcus luteus cells by penicillin lysis and by resuscitation in cell-free spent culture medium at high dilution." FEMS (FEDERATION OF EUROPEAN MICROBIOLOGICAL SOCIETIES) MICROBIOLOGY LETTERS 115 (2-3). 1994. 347-352. ISSN: 0378-1097, XP002080582	33
Y	see the whole document	1-10, 15-18, 44,47

A	KAPRELYANTS A S ET AL: "Do bacteria need to communicate with each other for growth?." TRENDS IN MICROBIOLOGY, (1996 JUN) 4 (6) 237-42. REF: 70 JOURNAL CODE: B1N. ISSN: 0966-842X., XP002080583 ENGLAND: United Kingdom	

P,X	MURPHY L ET AL:'Hypothetical 38.1 KD protein.' TRPRO Database entry 005594, 01-Jul-1997 Accession number 005594 XP002080584 see sequence	1-10, 15-18, 30,33,47

P,X	BADCOCK K ET AL: 'Hypothetical 17.7 KD protein.' TRPRO Database entry 033049, 01-Jan-1998 Accession number 033049 XP002080585 see sequence	1-10, 15-18, 30,33,47

P,X	OLIVER K ET AL: 'Hypothetical 18.0 KD Protein.' TRPRO Database entry 007747, 01-Jul-1997 Accession number 007747 XP002080586 see sequence	1-10, 15-18, 30,33,47

X	DEVLIN K ET AL: 'Hypothetical 15.7 KD protein' TRPRO Database entry P71755, 01-Feb-1997 Accession number P71755 XP002080587 see sequence	1-10, 15-18, 30,33,47

	-/--	

3

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	OGASAWARA N ET AL: 'Hypothetical 47.7.KD protein in mets-ksga intergenic region.' SWISSPROT Database entry Yabe-Bacsu, 01-Oct-1994, Accession number P37546 XP002080588 cited in the application see sequence ---	1-10, 15-18, 30,33,47
P,X	KUNST F ET AL: 'Cell wall-binding protein homolog yocH-Bacillus subtilis' PIR Database entry E69901, 05-Dec-1997, Accession number E69901 XP002080589 see sequence ---	1-10, 15-18, 30,33,47
X	LYRISTIS M et al: 'Hypothetical protein 1 -Clostridium perfringens (fragment).' PIR Database entry S49552, 29-Nov-1995, Accession number S49552 XP002080590 see sequence ---	1-10, 15-18, 30,33,47
X	PARKER WD ET AL: 'Cytochrome oxidase mutations aiding diagnosis of sporadic alzheimer's disease.' EMPATENT Database entry I27393, 14-Feb-1997, Accession number I27393 XP002080591 see sequence -----	34

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 98/01619

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 23-29,39,50,52
because they relate to subject matter not required to be searched by this Authority, namely:
see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Claims Nos.: 23-29,39,50,52

Said claims relate to antagonists, inhibitors, agonists, activators, mimetics, antimicrobial drugs, biomolecules, and microorganisms without giving a true technical characterization of the claimed matter. In consequence, the scope of said claims is ambiguous and, moreover, their subject-matter is vague and not sufficiently disclosed.

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

United States Patent and Trademark Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 01 February 1999 (01.02.99)	
International application No. PCT/GB98/01619	**Applicant's or agent's file reference** P57398P
International filing date (day/month/year) 03 June 1998 (03.06.98)	**Priority date** (day/month/year) 04 June 1997 (04.06.97)
Applicant MUKAMOLOVA, Galina V. et al	

1. The designated Office is hereby notified of its election made:

   [X] in the demand filed with the International Preliminary Examining Authority on:

   23 December 1998 (23.12.98)

   [ ] in a notice effecting later election filed with the International Bureau on:

   _____

2. The election [X] was

   [ ] was not

   made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer S. Mafla
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

PRICE, Vincent Andrew.
FRY HEATH & SPENCE
The Old College
53 High Street
Horley, Surrey RH6 7BN
GRANDE BRETAGNE

RECEIVED
15 SEP 1999

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	14.09.99
Applicant's or agent's file reference P57398P	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB98/01619	03/06/1998	04/06/1997

Applicant
THE UNIVERSITY OF WALES et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Vullo, C Tel.+49 89 2399-8061

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
P57398P		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB98/01619	03/06/1998	04/06/1997
International Patent Classification (IPC) or national classification and IPC C12N15/31		
Applicant THE UNIVERSITY OF WALES et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 2 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☒ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
23/12/1998	14.09.99
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Fotaki, M. Telephone No. +49 89 2399 8709

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB98/01619

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-41	as originally filed			

Claims, No.:

1-15	as received on	20/08/1999	with letter of	18/08/1999

Drawings, sheets:

1/20-20/20	as originally filed

2. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

II. Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

   ☐ copy of the earlier application whose priority has been claimed.

   ☐ translation of the earlier application whose priority has been claimed.

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:

see separate sheet

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims 1-15
	No:	Claims
Inventive step (IS)	Yes:	Claims 1-15
	No:	Claims
Industrial applicability (IA)	Yes:	Claims 1-15
	No:	Claims

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

II. PRIORITY

1) This international preliminary examination report has been established considering the priority date 04.06.97 as a valid date. The Applicant is reminded that document:
KUNST F ET AL: 'Cell wall-binding protein homolog yocH-Bacillus subtilis' PIR Database entry E69901, 05-Dec-1997, Accession number E69901
cited in the international search report may become relevant after consideration of the priority document which is unavailable at present.

V. REASONED STATEMENT UNDER RULE 66.2 (a) (ii)

2) The present application relates to the purification of a resuscitation promoting factor (RP-factor) from Micrococcus luteus as well as the identification of a gene which encodes said RP-factor. The amino acid and nucleotide sequence of the identified gene is presented in Figure 2. Homology search through a sequence database revealed several genes with previously unknown function which share extensive homology with the M. luteus RP-factor gene. These genes are found in high G+C Gram-positive bacteria such as M. tuberculosis , Mycobacterium leprae and Streptomyces coelicolor (9 sequences in Figure 1A) and in low G+C Gram-positive bacteria such as Bacillus subtilis, Clostridium acetobutylicum, and Clostridium perfringens (5 sequences in Figure 1B part B). Said RP-like sequences are expected to represent RP-factor homologues or species variants.

The purified RP-factor from M. luteus was shown to be capable of resuscitating of dormant cells (return to culturable state) of M. luteus and M. tuberculosis. Furthermore, M. luteus RP-factor was shown to be capable of growth stimulating activity of cells by shortening the apparent lag phase of batch cultures of several species of mycobacteria. Recombinantly expressed RP-factor (in E. coli) was used for the production of antibodies which inhibited the growth of a M. luteus culture.

Document (D1) KAPRELYANTS A S ET AL: in TRENDS IN MICROBIOLOGY, (1996 JUN) 4 (6) 237-42, which is considered to represent the closest prior art document discloses evidence for the involvement of hormones and pheromones

in prokaryotic growth and division (p.237). Addition of supernatants from growing M. luteus cultures to the starved culture relieves the inability of the cells to divide more than a few times and allows resuscitation of cells to normal colony-forming cells (p. 238). Table 1 of the same document lists some autocrine (pheromone) substances that stimulate the growth of bacteria, among which is listed a substance of unknown chemical nature, probably a protein, derived from M. luteus capable of resuscitation and stimulation of growth. Given the complexity of the supernatants of bacterial cultures and the numerous candidate substances present therein, discussed in the same document, the identification of a defined polypeptide capable for resuscitating bacterial cells is not considered to be anticipated nor obvious. Thus, the subject-matter of **Claims 1-15** is considered novel and inventive as required by Article 33(2) and (3) PCT.

VIII. CERTAIN OBSERVATIONS ON THE INTERNATIONAL APPLICATION

3) The subject-matter of **Claim 3** does not meet the requirements of Rule 6 for clarity. The term "Rpf factor" should appear in full name with the abbreviated form next to it, in brackets.

CLAIMS:

1. An isolated polypeptide capable of resuscitating dormant, moribund or latent bacterial cells, which polypeptide comprises: (i) a sequence of amino acid residues wherein the identities and relative positions of amino acid residues therein correspond to the residues indexed by asterisks in any one of the sequences set out in Figure 1A or Figure 1B(B), or (ii) a sequence which has at least 20% identity or homology with the sequence defined in (i).

2. The polypeptide of claim 1 which is any one of the polypeptides represented in Figure 1A or Figure 1B, or a homologue, allelic form, species variant or mutein thereof.

3. The polypeptide of claim 1 which is the *M. luteus* Rpf factor represented in Fig. 2A, or a homologue, allelic form, species variant or mutein thereof.

4. The polypeptide of any one of the preceding claims which is recombinant.

5. A pharmaceutical composition (e.g. a vaccine) comprising the polypeptide of any one of the preceding claims.

6. The polypeptide of any one of claims 1 to 4 which is:

(a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or

(b) in a pharmaceutical excipient, a unit dosage form or in a form suitable for local or systemic administration.

7. An antibody (or antibody derivative) specific for the polypeptide of any one of claims 1 to 4.

8. The antibody of claim 7 which is:

(a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or

(b) in a pharmaceutical excipient, a unit dosage form or in a form suitable for local or systemic administration.

9. Isolated nucleic acid encoding the polypeptide defined in any one of claims 1 to 4.

10. A vector (e.g. an expression vector) comprising the nucleic acid of claim 9.

11. A host cell comprising the vector of claim 10.

12. The nucleic acid of claim 9 or vector of claim 10 in a pharmaceutical excipient.

13. A diagnostic kit, culture medium or transport medium comprising the polypeptide of any one of claims 1 to 4.

14. An *ex vivo* method of diagnosis, comprising the step of contacting a biological sample with the polypeptide of any one of claims 1 to 4.

15. A live vaccine comprising an attenuated microbe, which microbe bears a mutation in a gene encoding (or regulating the expression of) the polypeptide defined in any one of claims 1 to 4.

19

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
P57398P		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB98/01619	03/06/1998	04/06/1997
International Patent Classification (IPC) or national classification and IPC C12N15/31		
Applicant THE UNIVERSITY OF WALES et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 2 sheets.

3. This report contains indications relating to the following items:

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   - II ☒ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
23/12/1998	14.09.99
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Fotaki, M Telephone No. +49 89 2399 8709

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB98/01619

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

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1-41	as originally filed				

Claims, No.:

1-15	as received on	20/08/1999	with letter of	18/08/1999	

Drawings, sheets:

1/20-20/20	as originally filed

2. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

II. Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

   ☐ copy of the earlier application whose priority has been claimed.

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2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:

see separate sheet

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-15
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-15
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-15
	No:	Claims	

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

II. PRIORITY

1) This international preliminary examination report has been established considering the priority date 04.06.97 as a valid date. The Applicant is reminded that document:
KUNST F ET AL: 'Cell wall-binding protein homolog yocH-Bacillus subtilis' PIR Database entry E69901, 05-Dec-1997, Accession number E69901
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V. REASONED STATEMENT UNDER RULE 66.2 (a) (ii)

2) The present application relates to the purification of a resuscitation promoting factor (RP-factor) from Micrococcus luteus as well as the identification of a gene which encodes said RP-factor. The amino acid and nucleotide sequence of the identified gene is presented in Figure 2. Homology search through a sequence database revealed several genes with previously unknown function which share extensive homology with the M. luteus RP-factor gene. These genes are found in high G+C Gram-positive bacteria such as M. tuberculosis , Mycobacterium leprae and Streptomyces coelicolor (9 sequences in Figure 1A) and in low G+C Gram-positive bacteria such as Bacillus subtilis, Clostridium acetobutylicum, and Clostridium perfringens (5 sequences in Figure 1B part B). Said RP-like sequences are expected to represent RP-factor homologues or species variants.

The purified RP-factor from M. luteus was shown to be capable of resuscitating of dormant cells (return to culturable state) of M. luteus and M. tuberculosis. Furthermore, M. luteus RP-factor was shown to be capable of growth stimulating activity of cells by shortening the apparent lag phase of batch cultures of several species of mycobacteria. Recombinantly expressed RP-factor (in E. coli) was used for the production of antibodies which inhibited the growth of a M. luteus culture.

Document (D1) KAPRELYANTS A S ET AL: in TRENDS IN MICROBIOLOGY, (1996 JUN) 4 (6) 237-42, which is considered to represent the closest prior art document discloses evidence for the involvement of hormones and pheromones

in prokaryotic growth and division (p.237). Addition of supernatants from growing M. luteus cultures to the starved culture relieves the inability of the cells to divide more than a few times and allows resuscitation of cells to normal colony-forming cells (p. 238). Table 1 of the same document lists some autocrine (pheromone) substances that stimulate the growth of bacteria, among which is listed a substance of unknown chemical nature, probably a protein, derived from M. luteus capable of resuscitation and stimulation of growth. Given the complexity of the supernatants of bacterial cultures and the numerous candidate substances present therein, discussed in the same document, the identification of a defined polypeptide capable for resuscitating bacterial cells is not considered to be anticipated nor obvious. Thus, the subject-matter of **Claims 1-15** is considered novel and inventive as required by Article 33(2) and (3) PCT.

VIII. CERTAIN OBSERVATIONS ON THE INTERNATIONAL APPLICATION

3) The subject-matter of **Claim 3** does not meet the requirements of Rule 6 for clarity. The term "Rpf factor" should appear in full name with the abbreviated form next to it, in brackets.

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* P57398P

Box No. I TITLE OF INVENTION

BACTERIAL PHEROMONES AND USES THEREFOR

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

THE UNIVERSITY OF WALES,
Aberystwyth, Old College
King Street
Aberystwyth SY23 2AX
GB

☐ This person is also inventor.

Telephone No.

Facsimile No.

Teleprinter No.

State *(i.e. country)* of nationality: GB

State *(i.e. country)* of residence: GB

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

MUKAMOLOVA, Galina V.
uL. Oktyabrskaya d29 kv392
g. Zheleznodorozhnii
Moscow Region
Russia

This person is:
☐ applicant only
☒ applicant and inventor
☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: RU

State *(i.e. country)* of residence: RU

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

PRICE, Vincent Andrew
Fry Heath & Spence
The Old College
53 High Street
Horley
Surrey RH6 7BN, GB

Telephone No. +44 1293 776880

Facsimile No. +44 1293 776837

Teleprinter No.

☐ Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Continuation of Box No. III FURT[redacted] APPLICANTS AND/OR (FURTHER) INV[redacted]RS

If none of the following sub-boxes is used, this sheet is not to be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

KAPRELYANTS, Arseny S.

Academician Anochin Street 38/1 Apt. 28
117602 Moscow
Russia

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: RU

State *(i.e. country)* of residence: RU

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

YOUNG, Danielle I.

Bell Vue
Llanilar
Ceredigion SY23 4PG
GB

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: GB

State *(i.e. country)* of residence: GB

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

KELL, Douglas B.

Symlog House
Cwm Symlog
Ceredigion SY23 3HA
GB

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: GB

State *(i.e. country)* of residence: GB

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

YOUNG, Michael

Belle Vue
Llanilar
Ceredigion SY23 4PG
GB

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: GB

State *(i.e. country)* of residence: GB

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on another continuation sheet.

Box No.V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes; at least one must be marked)*:

Regional Patent

- ☒ **AP** ARIPO Patent: GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, SD Sudan, SZ Swaziland, UG Uganda, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
- ☒ **EA** Eurasian Patent: AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
- ☒ **EP** European Patent: AT Austria, BE Belgium, CH and LI Switzerland and Liechtenstein, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- ☒ **OA** OAPI Patent: BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*:

- ☒ AL Albania
- ☒ AM Armenia
- ☒ AT Austria
- ☒ AU Australia
- ☒ AZ Azerbaijan
- ☒ BA Bosnia and Herzegovina
- ☒ BB Barbados
- ☒ BG Bulgaria
- ☒ BR Brazil
- ☒ BY Belarus
- ☒ CA Canada
- ☒ CH and LI Switzerland and Liechtenstein
- ☒ CN China
- ☒ CU Cuba
- ☒ CZ Czech Republic
- ☒ DE Germany
- ☒ DK Denmark
- ☒ EE Estonia
- ☒ ES Spain
- ☒ FI Finland
- ☒ GB United Kingdom
- ☒ GE Georgia
- ☒ GH Ghana
- ☒ GM Gambia
- ☒ GW Guinea-Bissau
- ☒ HU Hungary
- ☒ ID Indonesia
- ☒ IL Israel
- ☒ IS Iceland
- ☒ JP Japan
- ☒ KE Kenya
- ☒ KG Kyrgyzstan
- ☒ KP Democratic People's Republic of Korea
- ☒ KR Republic of Korea
- ☒ KZ Kazakhstan
- ☒ LC Saint Lucia
- ☒ LK Sri Lanka
- ☒ LR Liberia
- ☒ LS Lesotho
- ☒ LT Lithuania
- ☒ LU Luxembourg
- ☒ LV Latvia
- ☒ MD Republic of Moldova
- ☒ MG Madagascar
- ☒ MK The former Yugoslav Republic of Macedonia
- ☒ MN Mongolia
- ☒ MW Malawi
- ☒ MX Mexico
- ☒ NO Norway
- ☒ NZ New Zealand
- ☒ PL Poland
- ☒ PT Portugal
- ☒ RO Romania
- ☒ RU Russian Federation
- ☒ SD Sudan
- ☒ SE Sweden
- ☒ SG Singapore
- ☒ SI Slovenia
- ☒ SK Slovakia
- ☒ SL Sierra Leone
- ☒ TJ Tajikistan
- ☒ TM Turkmenistan
- ☒ TR Turkey
- ☒ TT Trinidad and Tobago
- ☒ UA Ukraine
- ☒ UG Uganda
- ☒ US United States of America
- ☒ UZ Uzbekistan
- ☒ VN Viet Nam
- ☒ YU Yugoslavia
- ☒ ZW Zimbabwe

Check-boxes reserved for designating States (for the purposes of a national patent) which have become party to the PCT after issuance of this sheet:

- ☐ .....
- ☐ .....
- ☐ .....

In addition to the designations made above, the applicant also makes under Rule 4.9(b) all designations which would be permitted under the PCT except the designation(s) of _____

The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)*

Supplemental Box *If the Supplemental Box is not used, this sheet need not be included in the request.*

Use this box in the following cases:

1. If, in any of the Boxes, the space is insufficient to furnish all the information:

in particular:

(i) if more than two persons are involved as applicants and/or inventors and no "continuation sheet" is available:

in such case, write "Continuation of Box No. ..." [indicate the number of the Box] and furnish the information in the same manner as required according to the captions of the Box in which the space was insufficient;

in such case, write "Continuation of Box No. III" and indicate for each additional person the same type of information as required in Box No. III. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below;

(ii) if, in Box No. II or in any of the sub-boxes of Box No. III, the indication "the States indicated in the Supplemental Box" is checked:

in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the applicant(s) involved and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is applicant;

(iii) if, in Box No. II or in any of the sub-boxes of Box No. III, the inventor or the inventor/applicant is not inventor for the purposes of all designated States or for the purposes of the United States of America:

in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the inventor(s) and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is inventor;

(iv) if, in addition to the agent(s) indicated in Box No. IV, there are further agents:

in such case, write "Continuation of Box No. IV" and indicate for each further agent the same type of information as required in Box No. IV;

(v) if, in Box No. V, the name of any State (or OAPI) is accompanied by the indication "patent of addition," or "certificate of addition," or if, in Box No. V, the name of the United States of America is accompanied by an indication "Continuation" or "Continuation-in-part":

in such case, write "Continuation of Box No. V" and the name of each State involved (or OAPI), and after the name of each such State (or OAPI), the number of the parent title or parent application and the date of grant of the parent title or filing of the parent application;

(vi) if there are more than three earlier applications whose priority is claimed:

in such case, write "Continuation of Box No. VI" and indicate for each additional earlier application the same type of information as required in Box No. VI.

2. If the applicant claims, in respect of any designated Office, the benefits of provisions of the national law concerning non-prejudicial disclosures or exceptions to lack of novelty:

in such case, write "Statement Concerning Non-Prejudicial Disclosures or Exceptions to Lack of Novelty" and furnish that statement below.

Continuation of Box No. IV

FRY, Alan Valentine; HUTCHINS, Michael Richard; SPENCE, Anne; DOWNING, Michael Philip; all of Fry Heath & Spence, The Old College, 53 High Street, Horley, Surrey RH6 7BN, GB

Box No. VI PRIORITY CLAIM Further priority claims are [illegible]ed in the Supplemental Box ☐

The priority of the following earlier application(s) is hereby claimed:

Country *(in which, or for which, the application was filed)*	Filing Date *(day/month/year)*	Application No.	Office of filing *(only for regional or international application)*
item (1) GB	4 June 1997 (04.06.1997)	9711389.8	
item (2) GB	27 May 1998 (27.05.1998)	9811221.2	
item (3)			

Mark the following check-box if the certified copy of the earlier application is to be issued by the Office which for the purposes of the present international application is the receiving Office (a fee may be required):

☒ The receiving Office is hereby requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) identified above as item(s): (1) and (2)

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(If two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):* ISA /

Earlier search *Fill in where a search (international, international-type or other) by the International Searching Authority has already been carried out or requested and the Authority is now requested to base the international search, to the extent possible, on the results of that earlier search. Identify such search or request either by reference to the relevant application (or the translation thereof) or by reference to the search request:*

Country (or regional Office): Date *(day/month/year)*: Number:

Box No. VIII CHECK LIST

This international application contains the following number of sheets:

1. request	5	sheets
2. description	41	sheets
3. claims	7	sheets
4. abstract	1	sheets
5. drawings	20	sheets
Total	74	sheets

This international application is accompanied by the item(s) marked below:

1. ☐ separate signed power of attorney
2. ☐ copy of general power of attorney
3. ☐ statement explaining lack of signature
4. ☐ priority document(s) *identified in Box No. VI as item(s):*
5. ☒ fee calculation sheet
6. ☐ separate indications concerning deposited microorganisms
7. ☐ nucleotide and/or amino acid sequence listing (diskette)
8. ☐ other *(specify)*:

Figure No. _____ of the drawings (if any) should accompany the abstract when it is published.

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

.....
V.A. Price

For receiving Office use only

1. Date of actual receipt of the purported international application:
2. Drawings: ☐ received: ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:
4. Date of timely receipt of the required corrections under PCT Article 11(2):
5. International Searching Authority specified by the applicant: ISA /
6. ☐ Transmittal of search copy delayed until search fee is paid

For International Bureau use only

Date of receipt of the record copy by the International Bureau:

This sheet is not part [redacted] and does not count as a sheet of the internat[redacted] application.

PCT

FEE CALCULATION SHEET

Annex to the Request

For receiving Office use only

International application No.

Date stamp of the receiving Office

Applicant's or agent's file reference P57398P

Applicant UNIVERSITY OF WALES et al

CALCULATION OF PRESCRIBED FEES

1. TRANSMITTAL FEE . . . . . £ 55.00 [T]

2. SEARCH FEE . . . . . £753.00 [S]

International search to be carried out by _____

(If two or more International Searching Authorities are competent in relation to the international application, indicate the name of the Authority which is chosen to carry out the international search.)

3. INTERNATIONAL FEE

Basic Fee

The international application contains 74 sheets.

first 30 sheets . . . . . £ 285.00 [b_1]

44 (remaining sheets) x £6 (additional amount) = £ 264.00 [b_2]

Add amounts entered at b_1 and b_2 and enter total at B . . . . £549.00 [B]

Designation Fees

The international application contains all designations.

11 (number of designation fees payable *(maximum 11)*) x £65 (amount of designation fee) = £715.00 [D]

Add amounts entered at B and D and enter total at I . . . . . £1,264.00 [I]

(Applicants from certain States are entitled to a reduction of 75% of the international fee. Where the applicant is (or all applicants are) so entitled, the total to be entered at I is 25% of the sum of the amounts entered at B and D.)

4. FEE FOR PRIORITY DOCUMENT . . . . . £ 44.00 [P]

5. TOTAL FEES PAYABLE

Add amounts entered at T, S, I and P, and enter total in the TOTAL box . . £2,116.00 TOTAL

☐ The designation fees are not paid at this time.

MODE OF PAYMENT

- ☐ authorization to charge deposit account (see below)
- ☒ cheque
- ☐ postal money order
- ☐ bank draft
- ☐ cash
- ☐ revenue stamps
- ☐ coupons
- ☐ other *(specify):*

DEPOSIT ACCOUNT AUTHORIZATION *(this mode of payment may not be available at all receiving Offices)*

The RO/ _____

- ☐ is hereby authorized to charge the total fees indicated above to my deposit account.
- ☐ is hereby authorized to charge any deficiency or credit any overpayment in the total fees indicated above to my deposit account.
- ☐ is hereby authorized to charge the fee for preparation and transmittal of the priority document to the International Bureau of WIPO to my deposit account.

Deposit Account Number _____ Date *(day/month/year)* _____ Signature _____

See Notes to the fee calculation sheet

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
P57398P		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/GB 98/ 01619	03/06/1998	04/06/1997
Applicant		
THE UNIVERSITY OF WALES et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 7 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. [X] **Certain claims were found unsearchable** (see Box I).

2. [ ] **Unity of invention is lacking** (see Box II).

3. [X] The international application contains disclosure of a **nucleotide and/or amino acid sequence listing** and the international search was carried out on the basis of the sequence listing
   - [ ] filed with the international application.
   - [X] furnished by the applicant separately from the international application,
     - [ ] but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.
   - [ ] Transcribed by this Authority

4. With regard to the **title,**
   - [X] the text is approved as submitted by the applicant
   - [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**
   - [X] the text is approved as submitted by the applicant
   - [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this International Search Report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is:
   Figure No. —
   - [ ] as suggested by the applicant.
   - [ ] because the applicant failed to suggest a figure.
   - [ ] because this figure better characterizes the invention.
   - [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 98/01619

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 23-29,39,50,52
because they relate to subject matter not required to be searched by this Authority, namely:
see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Claims Nos.: 23-29,39,50,52

Said claims relate to antagonists, inhibitors, agonists, activators, mimetics, antimicrobial drugs, biomolecules, and microorganisms without giving a true technical characterization of the claimed matter. In consequence, the scope of said claims is ambiguous and, moreover, their subject-matter is vague and not sufficiently disclosed.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 98/01619

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/31 C07K14/195 C07K14/315 C07K14/31 C07K14/32
C07K14/33 C07K14/335 C07K14/305 C07K14/35 C07K14/36
C07K14/34 C12N1/38 A61K39/02 A61K39/05 A61K39/07

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K G01N C12Q

Documentation searched other than minimumdocumentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	MUKAMOLOVA G V ET AL: "A bacterial cytokine." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1998 JUL 21) 95 (15) 8916-21. JOURNAL CODE: PV3. ISSN: 0027-8424., XP002080580 United States see the whole document --- -/--	1-10, 15-18, 44,47

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publicationdate of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of theinternational search	Date of mailing of the international search report
22 October 1998	04/11/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Espen, J

3

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 98/01619

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/08 A61K39/085 C07K16/12 G01N33/50 C12Q1/68
C12N15/11

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VOTYAKOVA T V ET AL: "Influence of viable cells on the resuscitation of dormant cells in Micrococcus luteus cultures held in an extended stationary phase: The population effect." APPLIED AND ENVIRONMENTAL MICROBIOLOGY 60 (9). 1994. 3284-3291. ISSN: 0099-2240, XP002080581	33
Y	see the whole document	1-10, 15-18, 44,47
	--- -/--	

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 October 1998	

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Espen, J

3

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	KAPRELYANTS A S ET AL: "Estimation of dormant Micrococcus luteus cells by penicillin lysis and by resuscitation in cell-free spent culture medium at high dilution." FEMS (FEDERATION OF EUROPEAN MICROBIOLOGICAL SOCIETIES) MICROBIOLOGY LETTERS 115 (2-3). 1994. 347-352. ISSN: 0378-1097, XP002080582	33
Y	see the whole document	1-10, 15-18, 44,47

A	KAPRELYANTS A S ET AL: "Do bacteria need to communicate with each other for growth?." TRENDS IN MICROBIOLOGY, (1996 JUN) 4 (6) 237-42. REF: 70 JOURNAL CODE: B1N. ISSN: 0966-842X., XP002080583 ENGLAND: United Kingdom	

P,X	MURPHY L ET AL:'Hypothetical 38.1 KD protein.' TRPRO Database entry 005594, 01-Jul-1997 Accession number 005594 XP002080584 see sequence	1-10, 15-18, 30,33,47

P,X	BADCOCK K ET AL: 'Hypothetical 17.7 KD protein.' TRPRO Database entry 033049, 01-Jan-1998 Accession number 033049 XP002080585 see sequence	1-10, 15-18, 30,33,47

P,X	OLIVER K ET AL: 'Hypothetical 18.0 KD Protein.' TRPRO Database entry 007747, 01-Jul-1997 Accession number 007747 XP002080586 see sequence	1-10, 15-18, 30,33,47

X	DEVLIN K ET AL: 'Hypothetical 15.7 KD protein' TRPRO Database entry P71755, 01-Feb-1997 Accession number P71755 XP002080587 see sequence	1-10, 15-18, 30,33,47

	-/--	

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	OGASAWARA N ET AL: 'Hypothetical 47.7.KD protein in mets-ksga intergenic region.' SWISSPROT Database entry Yabe-Bacsu, 01-Oct-1994, Accession number P37546 XP002080588 cited in the application see sequence ---	1-10, 15-18, 30,33,47
P,X	KUNST F ET AL: 'Cell wall-binding protein homolog yocH-Bacillus subtilis' PIR Database entry E69901, 05-Dec-1997, Accession number E69901 XP002080589 see sequence ---	1-10, 15-18, 30,33,47
X	LYRISTIS M et al: 'Hypothetical protein 1 -Clostridium perfringens (fragment).' PIR Database entry S49552, 29-Nov-1995, Accession number S49552 XP002080590 see sequence ---	1-10, 15-18, 30,33,47
X	PARKER WD ET AL: 'Cytochrome oxidase mutations aiding diagnosis of sporadic alzheimer's disease.' EMPATENT Database entry I27393, 14-Feb-1997, Accession number I27393 XP002080591 see sequence -----	34